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Functional roles of DTX3L and ARTD9 in prostate cancer

Inaugural-Dissertation

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1 Summary / Zusammenfassung

1.1 Summary

Prostate cancer (PCa) is one of the leading causes of cancer related mortality and morbidity in the aging male population. The B-lymphoma and BAL-associated protein (BBAP) and Deltex (DTX)-3-like E3 ubiquitin ligase (DTX3L), was originally identified as a binding partner of the B-aggressive lymphoma-1 protein and diphtheria-toxin-like ADP-ribosyltransferase-9 (BAL1/ARTD9).
Here it is shown, that DTX3L and ARTD9 are both overexpressed in PCa cells. Together with ARTD8 (ADP-ribosyltransferase-8) they mediate proliferation, survival and chemo-resistance of PCa cells. The effects of DTX3L and ARTD9 are dependent on the signal transducer and activator of transcription factor 1 (STAT-1). STAT-1 is known as a tumor suppressor, activated by interferon γ (IFNγ). Its antitumor effects are mainly based on the activation of the transcription of the interferon responding factor 1 (IRF1). New reports showed that a constitutively activated STAT-1 leads to an IFNγ- and chemo-resistance of tumors and acts as an oncogene. Such a situation occurred also in the analyzed PCa cells. The presented study shows that DTX3L and ARTD9 repress the expression of IRF1.
DTX3L also mediates migration of PCa cells in a STAT-1 and STAT-3-dependent manner. However, migration is not dependent on IFNγ/IRF1.
Together, this study suggests that the combined inhibition of STAT-1, ARTD8, ARTD9 and/or DTX3L could increase the efficacy of chemotherapy or radiation treatment in prostate cancer.

1.2 Zusammenfassung

Prostatakrebs (PK) ist in der älteren, männlichen Bevölkerung eine häufige Krankheits- und Todesursache. Das B-Lymphom und BAL-1-assozierte Protein BBAP, eine Deltex (DTX)-3 ähnliche E3 Ubiquitinligase (DTX3L), wurde als Bindungspartner vom B-aggressive lymphoma-1 Protein (BAL1/ARTD9) identifiziert. Hier wird gezeigt, dass diese Proteine auch in PK-Zellen überexprimiert sind.
Sie beeinflussen zusammen mit ARTD8 (ADP-ribosyltransferase-8) die Proliferation, das Überleben und die Chemoresistenz der PK-Zellen. Diese Einflussnahmen von DTX3L und ARTD9, jedoch nicht von ARTD8, sind vom signal transducer and activator of transcription factor 1 (STAT-1) abhängig. Das von Interferon γ (IFNγ) aktivierte STAT-1 ist ein bekannter Tumorsuppressor. Seine Wirkung basiert mehrheitlich auf dessen Aktivierung vom interferon responding factor 1 (IRF1). Neue Studien zeigen jedoch auf, dass ein konstant aktiviertes STAT-1 zu einer Resistenz gegenüber INFγ führt. Daher kann der ursprüngliche Tumorsuppressor zum Onkogen werden. Dies trifft auch auf die untersuchten PK-Zellen zu, in welchen DTX3L und ARTD9 zudem die Expression von IRF1 hemmen.
DTX3L fördert auch die Migrationsfähigkeit der untersuchten Zellen. Dies ist ebenfalls ein von STAT-1 und zusätzlich auch von STAT-3 abhängiger Effekt, welcher jedoch keine Beeinflussung von IFNγ oder IRF1 erfährt.
Zusammengefasst zeigt diese Arbeit die Inhibierung von STAT-1, ARTD8, ARTD9 und/oder DTX3L als neues mögliches Ziel in der Therapie von PK auf.

2 Introduction

2.1 Cancer

To successfully create such a complex system like an entire body, all its cells have to be regulated. To organize that, the cells send, receive and interpret an elaborate set of extra- and intracellular signals [2].
Based on this system all the cells should behave properly – resting, dividing, proliferating, differentiating or going through apoptosis – as required by the system as a whole [2].
The causes of cancer are diverse, very complex, and not yet fully understood. DNA damages, which are not repaired properly, i.e. in the regions of tumor suppressor genes, can cause cancerous mutations [3,4]. If these cells bearing cancerous mutations are not subsequently eliminated by undergoing intrinsic cell death or extrinsically by the immune system, these cells can be eventually transformed to cancer cells with or without the combination of existing or subsequent accumulation of genetic faults within these cells [3,4]. At least six characteristic molecular mechanisms or mutational events underlying the malignant transformation have been described: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [3,4].
Cancer can be grouped in benign and malign neoplasia. If it is benign it will grow in an expansive way and will never form metastases. On the other hand malignant neoplasia will grow infiltrative and can form metastases, or both.
Indeed it is not easy for a cancer to form metastases, because it has to fulfill many conditions [5]:
First of all the cells have to be able to separate from the mass. In a second step the single cells have to be able to destroy connective tissue and move toward blood or lymphatic vessels. Once they arrive there, they have to survive the physical traumas within the blood flow. If they manage to adhere to the wall of a vessel by specifically expressed molecules, they will finally invade a new part of the body. At the new site there is a hard selection of cancer cells again. They have to adapt on a new setting, need to stimulate the angiogenesis and try to evade the immune system.

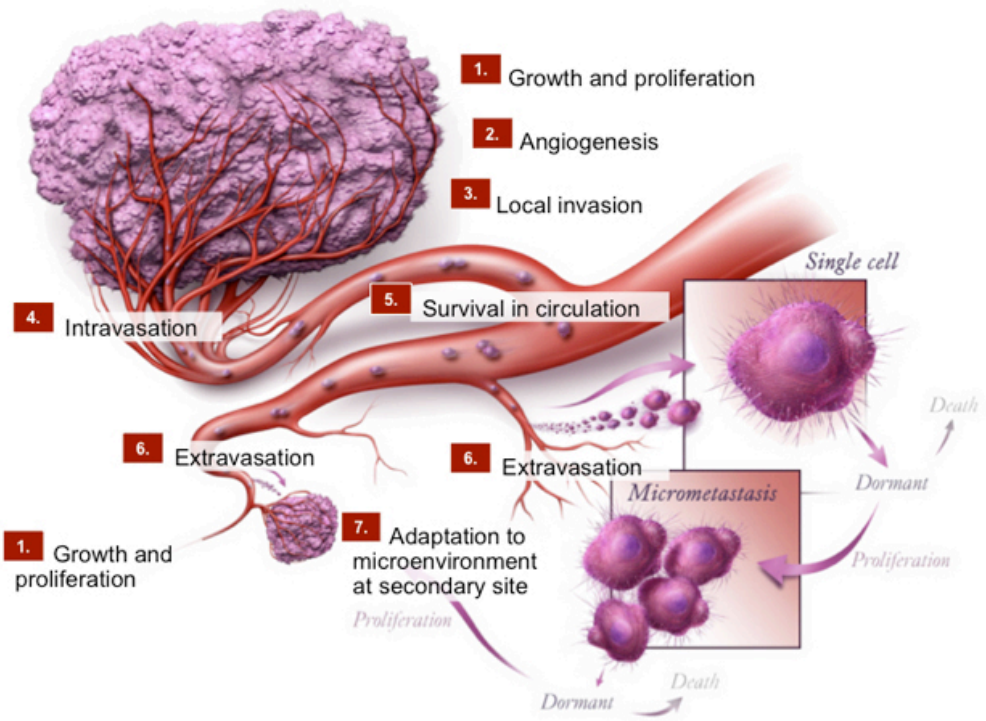

Figure 1: The different steps of forming metastases [6]. Cancer cells have to grow, proliferate, stimulate angiogenesis and be able to grow invasive. After the successful intravasation and survival in the circulation the cells have to extravasate and adapt to the new microenvironment.

2.1.1 Prostate carcinoma

Prostate carcinoma is the most common malignant neoplasia in men [7,8].
Known risk factors for this tumor are relatives who suffered from prostate carcinoma, but also the continent where someone lives and "age" [9, 10].
Studies from necropsies of over-90-year-old men (who had no cancer history) showed an incidence of prostate carcinoma of 70-100% [9]. It was shown by D. Ganten et. al. [10] that the highest incidence occurs in Afro-Americans living in the USA (137:100 000) and the lowest in China (2:100 000). It was also shown that the incidence increases in Asian people who immigrated to the USA [10]. But it is hard to say if this increase is due to a changed way of life or just a bias because of better screening methods.
Most prostate carcinomas are adenocarcinomas because typically the cancer cells have originally been glandular cells.
It is known that an up-regulated reaction to testosterone is a co-factor in the development of prostate cancer, but the causes are not yet clear [7]. Estrogen on the other hand acts as a protective factor [9].
At least 50% of prostate cancers show recurrent gene arrangement [11-13]. This mirrors the extensive biological variety in prostate cancer subtypes as well as the aggressiveness of the disease [11-13]. But also the subtypes have also some characteristics in common, like the dependency on an activated signal transducer and activator of transcription (STAT)-3 and NF-κB for survival [14].

Table 1: Overview of the different types of prostate cancer [1].

Prostate cancer type	Pathology	Frequency	Behavior
Prostatic intraepithelial neoplasia	Anaplastic cells between normal prostate glands		„Pre-stage“ of prostate cancer
Adenocarcinoma	Develops from prostate glands	>95%	- Local (85%): good prognosis - Infiltrative: bad prognosis - Metastatic: very bad prognosis
Small cell prostate cancer	Often in combination with adenocarcinoma	<1%	Highly aggressive, mean survival is 1 year
Mucin-producing carcinoma of the prostate	Develops from glands, produces mucosubstances	<1%	Highly aggressive, 3 year survival: 16.7%
Ductal prostate cancer	Involves the urethra and suburethral areas of the prostate. Develops from prostate duct epithelium	0.4%	No PSA (prostate specific antigen) production, often late diagnosis → Bad prognosis
Urothelium carcinoma	Develops from the ureter, renal pelvis or bladder	<1%	Highly aggressive
Squamous cell carcinoma	Forms often osteolytic bone metastases	<1%	Mean survival is 2 years Normal PSA concentrations

Clinical outcome and therapies

Due to the slow growth of prostate carcinomas and the frequent absence of clinical symptoms this disease is often diagnosed because of clinical signs of the metastases [9].
The clinical and molecular heterogeneity is still a great challenge in understanding and fighting prostate cancer [12-15]. Most cases of local and expansive growing prostate cancers are curable with surgical removal and radiation therapy [11,14]. About 80% of all prostate cancer cases are diagnosed in an early enough stage to be treated with mostly successful therapies. But the mortality rate of the other 20%, diagnosed as metastatic tumors, is still very high [14,16].
There are many possibilities for fighting prostate cancer, depending on the stage of the tumor: total surgical removal of the prostate, radiation, chemotherapy or a hormonal therapy (deficiency of androgens by antagonists of gonadotropin-releasing hormones or excision of the testes) [7].
Sadly, the hormonal therapy is usually not curative because after some time the prostate carcinomas develop an androgen-insensitivity [11]. This also marks the point in time when the cancer develops a more aggressive form and begins to form metastases [11].
The major problem in the therapy of androgen-independent prostate cancers still is the chemo-resistance [12-15].

2.2 Interferon and Signal Transducer and Activator of Transcription Factor 1 (STAT-1) pathway

Interferons build a family of secreted and pleiotropic cytokines which possess different kinds of antiproliferative, antiviral, immunomodulatory and antitumor effects [17-19]. They form three classes, also called type I, II and II IFN [17-19]:

Type I Interferons (IFN α and IFNβ): They are produced by nearly all the cells, which are infected by viruses but mainly by plasmacytoid dendritic cells. Their main effect is antiviral. They have just little immunomodulatory effects.
Type II Interferon (IFN γ): Only specialized T-cells and NK-cells do produce this interferon. Its main effect is immunomodulatory, weakly also antiviral.
Type III Interferon (IFN λ): Many cells produce this interferon. Its antiviral and immunomodulatory effects are weaker compared to the other types.

The biological effects of IFNs are mainly based on the activation of the signal transduction pathway of the Janus kinases (JAK1-4) and the signal transducers and activators of transcription (STAT) proteins, because they lead to the induction of IFN-dependent genes [20-22].
The interferons bind on specific receptors on the surface of the cell. That binding leads to the activation of the JAK (Janus kinases)/ STAT (Signal Tranducer and Activator of Transcription) pathway [17, 20, 23-24] (Figure 3):

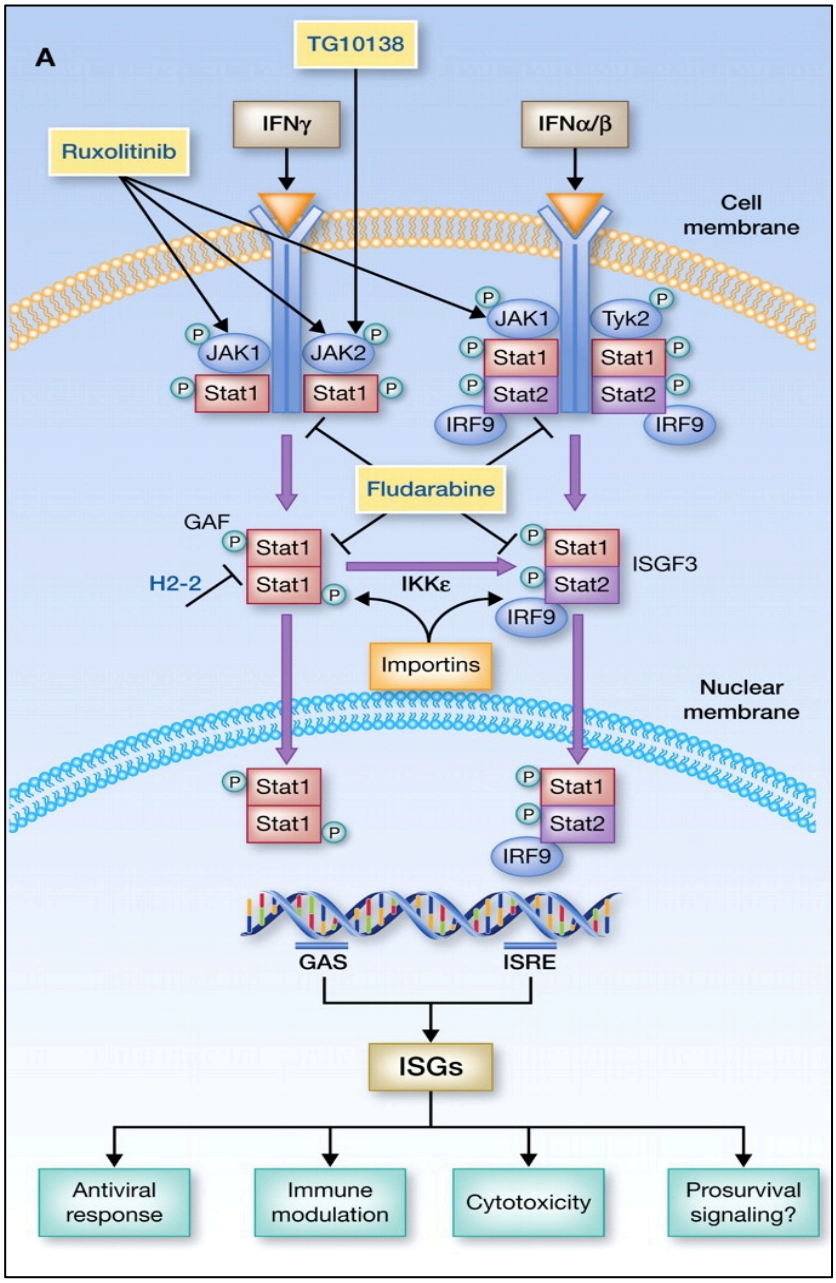

Figure 2: IFNγ/STAT-1 and IFNα/β/STAT-1/2 pathway [23]. After the binding of IFNγ on its receptor JAK1 and JAK2 get phosphorylated. This enables the phosphorylation of STAT-1, which then can form homodimers and in turn translocates into the nucleus. There it binds to the promotors of so-called interferon stimulated genes (ISGs) and stimulates their transcription.

The structural conformation of the receptor is changed after the binding of the interferon. This leads to the activation of JAK by phosphorylation. The activated JAK itself activates some STAT-family members. Activation of STAT family members is a highly specific process and is defined by the type of IFN and other activators.
In the case of IFN α or β it will be STAT-1 and STAT-2 that can form together with IRF9 (Interferon Responding Factor 9) a protein complex. This complex is also known as IFN-stimulated transcription factor gamma 3 (ISGF 3). Afterwards this protein complex is transported into the nucleus by importins to finally bind to the ISRE (IFN-stimulated Response Element) of so-called interferon stimulated genes (ISG) which leads to their transcription.

On the other hand if IFNγ activates the JAK/STAT pathway the result is a phosphorylated STAT-1 on Y701, which will form homodimers. They will translocate into the nucleus by specific importins where the homodimers will bind to the gamma-IFN-activated sequences (GAS). Therefore it will activate the transcription of other ISGs.
But STAT-1 can also be phosphorylated on S727, independently of the phosphorylation on Y701 [25]. This indicates that STAT-1 can also form heterodimers with other tyrosine phosphorylated STATs and therefore translocate into the nucleus in an Y701-independent manner [26, 27]. A possible STAT member is STAT-3, which can also be activated by INFγ in a cell type-specific manner [26-29].
One of the most rapidly activated IFN response genes is the transcription factor and interferon regulatory factor (IRF) 1, which itself enhances the transcription of several secondary response genes [30]. Together with IRF3 and IRF7, two other members of the interferon regulatory factor (IRF) family, IRF1 acts as one of the most important modulators of IFN dependent gene expression [30].

2.2.1 Chemo- and radioresistance in cancer and the IFN/STAT-1 signaling pathway

It is known that the interferons (IFNα, IFNβ as well as IFNγ) assume an important role in the coordination within the interactions between the immune system and tumors [31-33]. On the other hand the interferons do not only show antitumor properties but also protumor activities.
The coordination by interferons is regulated in two different ways:
Initially the IFNs protect the host against the development and formation of tumors (immunosurveillance) [34, 35]. For this goal IRF1 is one of the most important factors. IRF1 is not only an important factor of the innate immune system, but has also the ability of acting as tumor-suppressor in a context-dependent and cell-type specific manner [34]. Another important characteristic of IRF1 is the possibility of mediating anti-proliferating and pro-apoptotic effects in cancer cells [35]. Many tumors have a suppressed or reduced expression of IRF1 [34, 36].
But because of these anti-tumor effects of IFNs there is also the possibility of forcing the development of IFN-insensitive tumors (immunoediting) [528]. Due to the constant presence of IFNs in the microenvironment around the tumor cells, the insensitive ones will be selected [23].
It is known that these cells also have an upregulated STAT-1 and /or STAT-2 level [23].
Several recent studies strongly indicate that the transcription factor STAT-1 is involved in chemo and/or radiation resistance of solid tumors, including prostate cancer [23,37-43]. For instance, 29% of all clinical human prostate cancers, which were analyzed in a recent study, constitutively expressed STAT-1 and interferon-stimulated genes (ISGs) in vivo [23]. STAT-1 has therefore been suggested as a potential target for radio- and/or chemosensitization of aggressive tumors that constitutively overexpress IFN/STAT-1-dependent pathways. It has been shown that multiple low doses of ionizing radiation can activate an IFN (α, β and γ)-related, STAT-1-dependent DNA damage gene expression signature (including STAT-1, G1P2, G1P3, IFITM1, IFIT1, IRF9, MX1, HLA-C, OAS1 and OAS3) in prostate cancer [23, 37, 38, 42, 43]. It has even been shown that in vitro selection against IFN-α or IFN-γ and constitutive expression of STAT-1 leads to an IFN- and radioresistant phenotype in prostate tumor cells [23, 38]. In line with this observation,

the IFN/STAT-1 signaling pathway is also upregulated in chemoresistant prostate cancer cells [23, 42]. Remarkably, STAT-1-dependent chemo-resistance was also associated with increased resistance to ionizing radiation and the upregulation of ISGs that overlapped, in part, with the gene expression after IFN stimulation or DNA damage [23, 38]. Another evidence for the important role of STAT-1 in chemo- and radiation-resistance is the fact that hypoxic tumors generally do not respond to radiation [44]. It is known that STAT-1 is activated by hypoxia or re-oxygenation [44]. Moreover, it has been demonstrated for chemoresistant ovarian cancer cells that if JAK/STAT-1-signaling was inhibited, the sensitivity to some drugs increased significantly [40].

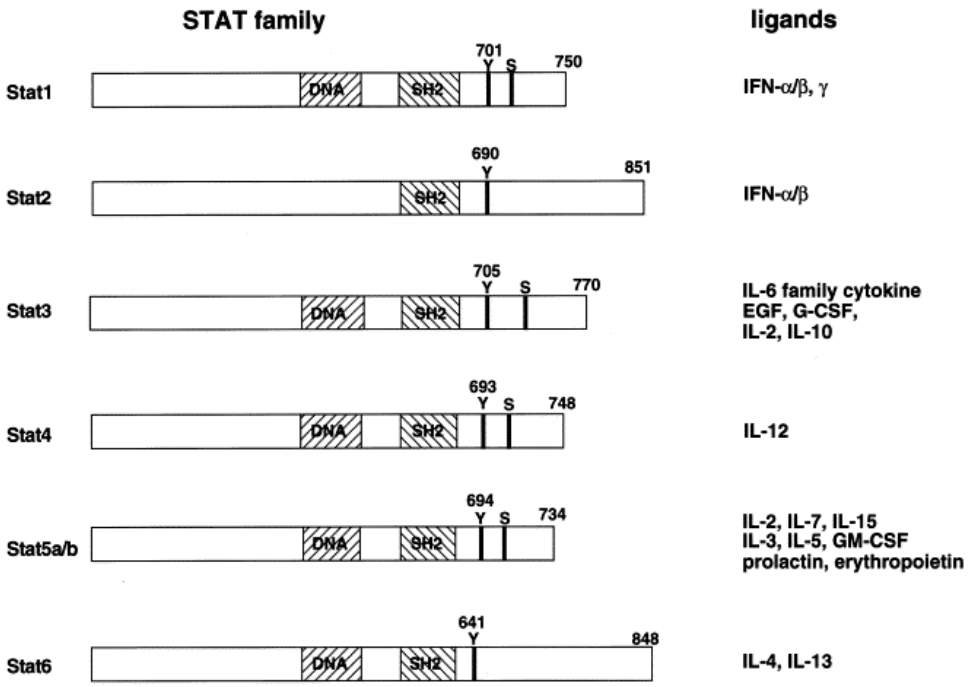

Figure 3: Overview of the STAT family members. DNA stands for DNA binging domain. SH2 is the region where the protein binds to its receptor or to another STAT-protein for dimerization. The conserved Tyrosin around 700 amino acid (Y) makes it possible for phosphorylated proteins to interact with the SH2 region of another protein. The most important ligands, which are capable of activating the STAT proteins are listed on the right side [45].

2.2.2 STAT-3; another crucial STAT-family member in tumorigenesis

STAT-3 plays important roles in tumor survival and growth and also in tumor migration, invasion and metastasis [26, 46].
STAT-3 can be activated by many agents [47], including IL-6, epidermal growth factor, Ras, but also by carcinogens like diesel exhaust particles [48] or cigarette smoke [47].
STAT-3 is activated by phosphorylation at either tyrosine 705 or serine 727 [47]. This phosphorylation is needed to form homodimers of STAT-3, which is then able to translocate into the nucleus [47]. In the nucleus it binds to the DNA and regulates the transcription of genes involved in inflammation, cell proliferation, differentiation, apoptosis and angiogenesis [47].
Singh et. al. [49] showed that STAT-3 (as well as STAT-5) is highly overexpressed in prostate cancer. Their study showed that expression of STAT-3 in cancer samples is highly elevated when compared to benign prostate hyperplasia (BHP) samples,

namely 68% compared to only 12% [49]. The same study also showed that in the cancer samples STAT-3 is more likely localized in the nucleus (89%) than in the BPH samples (9%).

2.3 B-aggressive lymphoma protein BAL-1/ARTD-9

The content of this paragraph is mainly based and modified from [1].
The risk-related B-agressive lymphoma protein BAL-1/ARTD9, as well as its highly related BAL-2/ARTD8 and BAL-3/ARTD7 family members [50, 51] are part of the intracellular diphtheria toxin-related family of mono- and polymerizing ADP-ribosyltransferases (ARTD) [52- 57]. The mammalian ARTD family consists of 18 (17) members (Figure 5).
Based on structural and enzymological data, the ARTD family has been divided into three mayor groups: (I) mono-ADP-ribosyltransferases, (II) polymerizing-ADP-ribosyltransferases and (III) most likely inactive mono-ADP-ribosyltransferase enzymes [50]. Mono- and poly-ADP-ribosylation of proteins are phylogenetically ancient and reversible processes [58, 59]. Their covalent post-translational modifications appear in a wide range of processes [58, 59].

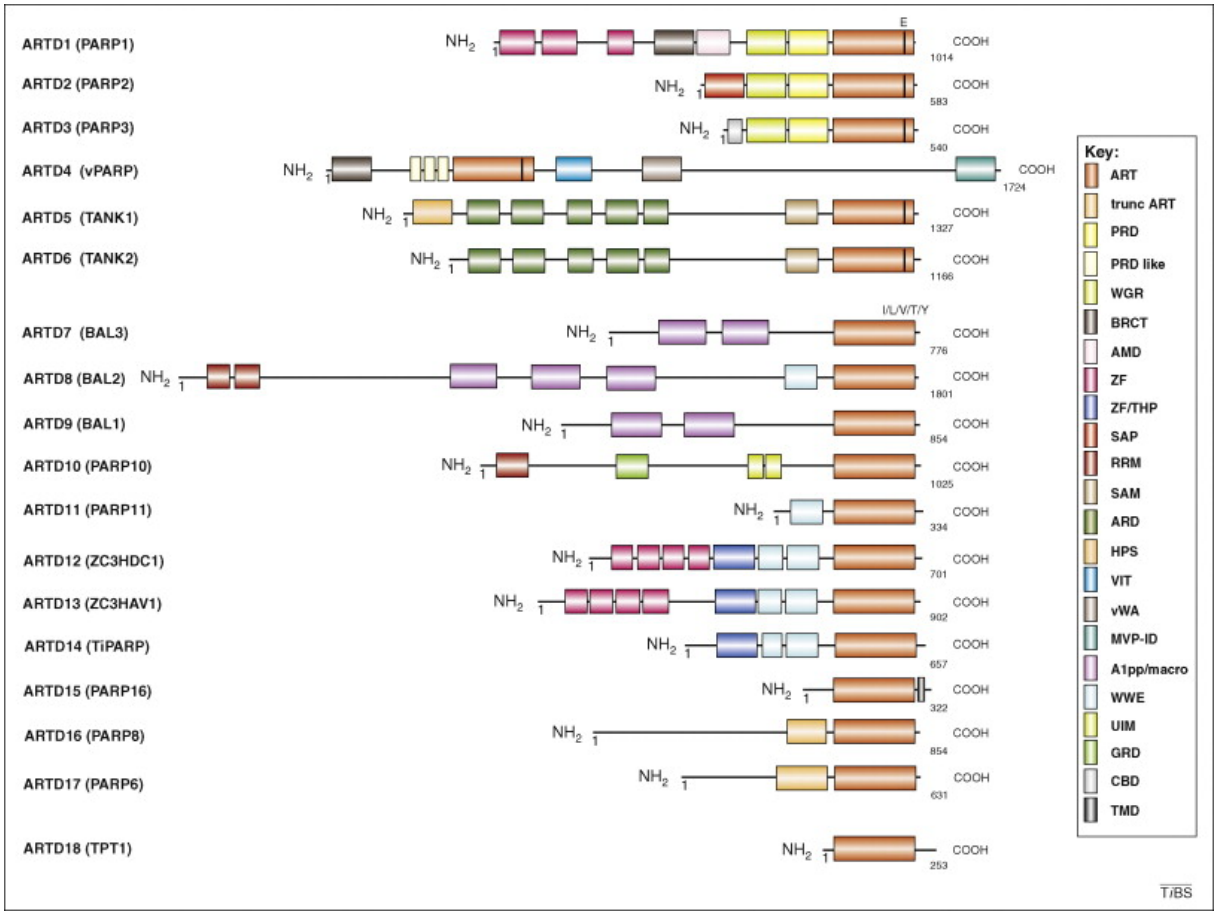

Figure 4: Schematic comparison of the domain architecture of the human ARTD (PARP) family [50]. ARTD7-9 are part of the most likely inactive mono-ADP-ribosyltransferase enzymes.

ARTD8 and ARTD7 are representatives of active mono-ADP-ribosyltransferases but so far no auto-modification or trans-mono-ADP-ribosyltransferase activity could be

shown for ARTD9 [60, 51]. Therefore, it is very likely that ARTD9 is an inactive ARTD.
All these three proteins exist in different isoforms. They contain either only one (ARTD9 and ARTD7) or two (ARTD8) macro domains [50].
Together with the DTX3L gene, all BAL genes are located on the same chromosome, in an evolutionary conserved gene cluster [50].
ARTD8 is suggested to be involved in mediating interleukin 4(IL-4)-dependent proliferation and protection of B-cells against apoptosis upon irritation or growth factor removal [54, 55, 61].
It has been previously demonstrated that ARTD9 acts as a novel oncogenic survival factor in high-risk, chemo-resistant, host response subtypes of diffuse large B-Cell lymphoma (HR-DLBCL) and as a crucial negative and positive co-regulator of IFNγ/STAT-1-signaling [72]. In addition ARTD9 has been shown to be activated by IFNγ/STAT-1 and IL6/STAT-3 signaling pathways.
The exact regulatory mechanisms and molecular functions of ARTD9 and its related ARTD8 and ARTD7 are only partially understood.

2.4 B-cell lymphoma and BAL-1-associated protein BBAP/DTX3L

The content of this paragraph is mainly based and modified from [1].
BBAP (B-cell lymphoma and BAL-1-associated protein) also named DTX3L was originally identified as a binding partner of ARTD9 [60, 52, 62]. DTX3L is a member of the E3 ligase family of DTX (Deltex) proteins [60]. The DTX protein family is defined by a typical buildup. The N-terminus with two WWE domains, allows binding to the Notch intracellular domain [63]. The central part of these proteins contains a proline-rich region that can bind to SH3 domain of specific proteins, however, a functionally binding partner in vivo has not yet been found [63, 62]. Due to the ring-H2 finger domain located at the C-terminal this protein family belongs to the E3 ubiquitin ligase family proteins [63, 62].
The Deltex family proteins do not only interact with Notch by their N-terminus but there are also indications that they can ubiquitinate Notch [63].
Notch itself has an influence on cancer, the immune system, aging and stem cell regulation [64]. It would therefore be interesting to better understand how DTX/Notch-like E3 ubiquitin ligases are regulated, especially with respect to therapeutic manipulations [64].
Figure 5 shows that DTX3L shares only the C-terminal part, i.e. the DTX catalytic domain, with the Deltex family. Therefore it is called Deltex 3-like protein.

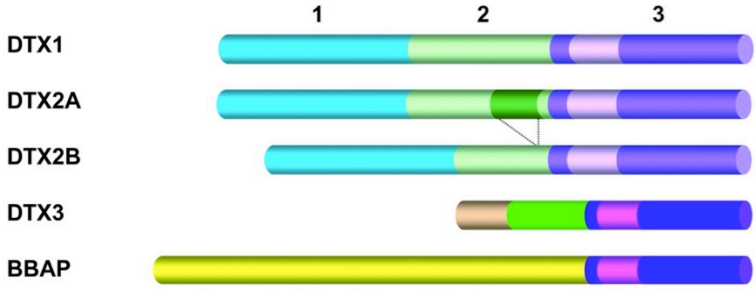

Figure 5: Domain organization of human DTX1, DTX2A, DTX2B, DTX3, and BBAP/DTX3L proteins. The N-terminal part is signed with 1, the central part with 2 and the C-terminal part with 3. It is visible that BBAP/DTX3L only shares the C-terminal part of DTX3 [62].

DTX3L is overexpressed in some high-risk chemotherapy-resistant diffuse large B-cell lymphomas (DLBCL), similar to ARTD9 [60,52].
DTX3L and also ARTD7-9 are highly inducible proteins. They are usually constitutively expressed only at extremely low levels in lymphocyte-rich tissues such as spleen, lymph nodes, peripheral blood lymphocytes, colonic mucosa and lung epithelial tissues [64, 65]. In adult mice, the highest expression level was found in the medulla of the thymus [64].
A bidirectional promotor which is highly responsive to IFNγ regulates the DTX3L gene [60]. ARTD9 is also controlled by this promotor [60].
Stimuation with IFNγ highly induces the expression of ARTD7-9. The same is true for IFNα/β and also for infection with H. pylori, S. typhimurium and various RNA viruses like Nipah virus or moderate and high virulent classical swine fever viruses [65-71].
It is not yet known if DTX3L also responds to factors other than IFNγ.
Yan et. al. [16] showed that DTX3L is able to selectively monoubiquitylate the histone H4 lysine 91 and also to protect cells exposed to DNA damaging agents. However, it remains to be investigated if DTX3L could also selectively monoubiquitinate signaling factors of the IFNγ or IFNα/β pathways such as ARTD9, STATs or IRF1.

2.5 Aim of the project

DTX3L was originally identified as a binding partner of ARTD9 [60, 52]. DTX3L and ARTD9, both also identified in a screen as novel risk-related gene products in HR-DLBCL [60, 52], are overexpressed in subtypes of high-risk chemotherapy-resistant aggressive HR-DLBCL with an active host inflammatory response and tightly associated with intrinsic IFNγ signaling and constitutive activity of STAT-1 [60, 52, 72]. Recent studies have provided first evidence that DTX3L and ARTD9 are also overexpressed in solid tumors [73,74]. However, the functions of DTX3L remain mostly unknown. Thus, a better understanding of this protein could help to combat cancer in a more efficient way or to find new strategies against cancer.
In this project we tried to shed light on the roles and influences of DTX3L in prostate cancer cells.
The main focus was placed on the understanding of the interference between DTX3L and the STAT-1 pathway, but also its interaction with its binding partner ARTD9 and the closely related ARTD8, known to play a role in tumorigenesis.
In addition we wanted to know whether DTX3L also functionally interacts with other STAT family members such as STAT-3 in cell survival, proliferation and cell migration of prostate cancer cells.

3 Material und Methods

3.1 Solutions

3.1.1 Protein extraction

Whole cell extraction (WCE):

50mM Tris, pH 7.5
Complete (protease inhibitor cocktail tablets), Roche®
1% NP-40
300mM NaCl

Bradford:

Dye concentrate (5x), Bio Rad®
ddH_2O
Protein standards (bovine serum albumin), Bio Rad®

3.1.2 SDS-pages and immunoblot solutions

10x SDS loading buffer:

20% SDS
600mM Tris, pH 6.8
20% Ficoll
0.05% bromphenol blue
20% mercaptoethanol
25mmol/l DTT
ddH_2O

Solution B:

1.5mol/l Tris-HCL pH 8.8
0.4% SDS

Solution C:

0.5mol/l Tris-HCL pH 6.8
0.4% SDS
small amount of bromphenol blue

Lower gel 10% SDS:

50%: ddH_2O
25%: Solution B
25%: acrylamide solution (40%)
1%: APS (10%)
0.1%: TEMED (tetramethylethylenediamine)

Upper gel 4.8% SDS:

63%: ddH_2O
25%: Solution C
12%: acrylamide solution (40%)
1%: APS (10%)
0.1%: TEMED (tetramethylethylenediamine)

10% APS (ammonium persulfate)

5x Running buffer:
- 125mmol/l Tris base
- 1mol/l glycine
- 0.5% SDS

Transfer buffer:
- 25mmol/l Tris base
- 192mmol/l glycine
- 800ml cold ddH_2O
- 200ml methanol

Blocking solution:
- Ready to use Odyssey-blocking-solution, diluted 1:2 in PBS, LI-COR®, Bioscience

10x TBS:
- 100mmol/l Tris-HCl pH 7.5
- 1500mmol/l NaCl
- ddH_2O to 1l

TBST:
- 100ml 10xTBS
- 0.5ml Tween 20
- ddH_2O to 1l

3.1.3 Cell fixation

10x PBS:
- 80g NaCl
- 2g KCl
- 14.4g Na_2HPO_4
- 2.4g KH_2PO_4
- adjusted to pH 6.8
- ddH_2O to 1l

Permeabilization buffer:
- 1% BSA
- 0.5% TRITON X-100
- PBS

Methanol

0.5% Tween 20 (in PBS)

Mounting media: VECTASHIELD® mounting media containing DAPI

3.1.4 Gene silencing

Opti-MEM® Reduced Serum Medium, Invitrogen®

Lipofectamine™ RNAiMAX Transfection Reagent, Invitrogen®

siRNA:
All the siRNA have a concentration of 40μM.

Table 2: Used siRNA sequences.

siRNA	Targeting sequence
AllStar mock siRNA negative control	GGGUAUCGACGAUUACAAA
ARTD9-siRNA #1	TGCAGGTTCTAAAGGTGGA
ARTD9-siRNA #2	GGCAAAGTCAATTCTACAA
ARTD9-siRNA #9	TTACCTTGGGTGAACTAAC
STAT-1-siRNA #6 (validated by Qiagen)	CAGAAAGAGCTTGACAGTAAA
STAT-1-siRNA #7 (validated by Qiagen)	CCAGATGTCTATGATCATTTA
ARTD8-siRNA #1	CTAGTGCAGATGTGTATAA
ARTD8-siRNA #2	GGA AAG GGC TCA CTC ACA ATT
DTX3L-siRNA #1	TCCAGGTTATGAGTCCTTTGGCA
DTX3L-siRNA #2	GTTAGAGGTGGGTCCGAAATAA
DTX3L-siRNA #3	GGCAAGCATTGGTAATAAATGGA
DTX3L-siRNA #4	GCCCTGCCACAGTAATGCTATA
STAT3-siRNA #7 (validated by Qiagen)	CAGCCTCTCTGCAGAATTCAA
STAT3-siRNA #8 (validated by Qiagen)	CAGGCTGGTAATTTATATAAT
IRF1-siRNA #1	CCAAGAACCAGAGAAAAGA
IRF1-siRNA #2	AGACCAGAGCAGGAACAAG

3.1.5 Antibodies

All antibodies were diluted in TBST containing 3% BSA and 1% sodiumazide for immunoblot analysis (WB) and in PBS containing 1% BSA for immunofluorescence (IF).

Anti-DTX3L:	US Biological, DTX3L, C12031405, rabbit, 1:5000 (WB), 1:100 (IF)
Anti-ARTD9:	Millipore, AB10618, polyclonal antibody, rabbit, 1: 200 (WB), 1:100 (IF)
Anti-STAT-1:	Epitomics, polyclonal antibody, rabbit, 1:1000 (IF)
Anti-pSTAT-1-Y701:	Epitomics, rabbit, 1:200 (IF)
Anti-pSTAT-1-S727:	Epitomics pS727, monoclonal antibody, rabbit, 1:500 (IF)
Anti-IRF1:	Cell Signaling, monoclonal antibody, rabbit, 1:500 (WB) Santa Cruz, rabbit, 1: 250 (WB)

Anti-IRF7: Epitomics, monoclonal antibody, rabbit, 1:1000 (WB)
Anti-Tubulin: Sigma, monoclonal antibody, mouse, 1:10'000 (WB)

Secondary antibodies:
- Immunoblot:
  Anti-rabbit: LI-COR, IRDye® 800CW goat anti-rabbit IgG, 1:15'000
  Anti-mouse: LI-COR, IRDye® 680RD goat anti-mouse IgG, 1: 15'000

- Immunofluorescence:
  Anti-rabbit: Invitrogen, Alexa Fluor 568 goat anti-rabbit IgG, 1:1000

3.1.6 RNA isolation

All the reagents are RNAase free.
TRI Reagent (Sigma® T-9424)
Ice cold PBS
Chloroform
75% Ethanol
Isopropanol
ddH_2O

3.1.7 Real time (RT)-PCR

'High-capacity cDNA reverse transcription' kit (Applied Biosystems®)

3.1.8 qPCR

SYBR Green kit (Bioline®)

Table 3: Used qPCR primers.

Target gene (human)	FWD primer	Rev primer
ARTD9	GGCAAAGAGGTCCAAGATGCTG	GCCTCACACATCTCTTCCACGT
DTX3L	CCAGGTTATGAGTCCTTTGGCAC	TGCAGTTCGCTGTATTCCAGGG
IRF1	AAAAGGAGCCAGATCCCAAGA	CATCCGGTACACTCGCACAG
GAPDH	GAAATCCCATCACCATCTTCC	GAGCCCCAGCCTTCTCCATG

3.1.9 Treatments
Human recombinant interferons were all purchased from PeproTech or kindly provided by Dr. J. Pavlovic (Institute of Medical Virology, University of Zurich, Switzerland), Docetaxel was purchased from SIGMA®.

3.2 Mammalian Cells

3.2.1 Culture conditions

All the cell lines were kept in a humified incubator at 37°C and 5%CO_2. The media were stored at 4°C and were preheated to 37°C before use. All the work was done under a sterile hood.
The medium was changed approximately every 3 days.
The calculation of the exact cell number was done by using an improved Neubauer counting chamber.

3.2.2 Cell lines and splitting of cells

PC3, DU145 and LNCaP
The metastatic prostate carcinoma cell lines PC3, DU145 and LNCaP were all from ATCC (American Type Culture Collection). They were cultured in 50% Ham's-F12 and 50% of RPMI, Glutamax-I, 10mmol/l HEPES with 10% FCS, and 10'000U/ml penicillin and streptomycin.
To split the cells the medium was discarded, the cells were washed in the 10cm plate with 4ml PBS. Afterwards 2ml trypsin was added and the cells were put in the incubator for 7min. 10ml medium was added to inhibit the trypsin, the cells were collected into a tube and centrifuged for 5min at 5000rpm. The supernatant was discarded and the cells were resuspended and diluted in fresh medium.

3.2.3 Preparation of whole cell extracts

The medium was discarded and PBS EDTA 10mM was added, 4ml in a 10cm plate respectively 1ml per well in a 6-well plate. The detached cells were centrifuged for 5min at 5000rpm. The supernatant was disposed and the pellet was resuspended with WCE buffer (3x the pellets volume). The cells were then incubated on ice for 25min. At 4°C the cells were centrifuged for 15min at 14'000rpm. The supernatant was collected.
For equal loadings of the SDS-gels, a Bradford was performed:
1ml of the 1x Bradford solution was prepared in a 1ml Eppendorf tube for one blank, four standards and each sample. Of each standard 10μl and of each sample 1-2μl were added to the corresponding tube. After vortexing and incubating the tubes at RT for 10min the fluids were transfused into cuvettes.
The photometric absorbance was measured with a Genesys 10uv scanning machine at 595nm wavelength.

3.2.4 siRNA transfection

500ml OptiMEM® medium with 4μl of each Lipofectamine® RNAiMAX and siRNA(40μM) were vortexed and incubated at RT for 20min. This mixture was added to 200'000 cells, which were diluted in 1ml of medium in 6-well plates.
After 48h in the incubator the cells were used for further experiments.

3.3 Detection methods

3.3.1 Immunoblot analysis

SDS-gels were always loaded with 60μg of protein. A prestained protein marker was always used as a protein size marker and loaded in the first slot. The gels ran at 100V for 15min and at 125V for 60-90min.
The proteins were transferred to Immobilon-FL transfer membranes (Millipore®) either for 1h with 100-120V at 4°C or ON with 30V at 4°C.
The membrane was blocked in blocking solution for 1h at RT or ON at 4°C.
The membrane was rinsed shortly in TBST and then stained with the diluted primary antibody for 2h at RT or ON at 4°C. After 3x5min of washing in TBST the membrane was stained in the dark with the diluted secondary antibody for 1h at RT.
The membrane was washed again 3x5min (in the dark) and then scanned by Odyssey® Infrared Imaging System.
For quantification of the signals GelEval® was used. Mean value ± SE was calculated and blotted into graphs with GraphPad Prism 5 software (GraphPad Software).

3.3.2 Immunofluorescence microscopy

200'000 cells per well were seeded on 22mm glass coverslips in 6-well plates.
If required, the cells were silenced for specific genes as described. If the cells were stimulated with interferon γ the concentration was 200U/ml. The interferons stimulated the cells for 2h in the incubator.
Afterwards the cells were fixed:
After the medium was discarded, 2ml of ice cold methanol was added to each well and the cells were stored for 20min at -20°C. The cells were washed 2x3min with PBS and permeabilized for 5min with 2ml 0.5% Tween 20. After 2x3min washing with PBS the cells were ready for staining.
For staining the coverslips were placed on 40μl of the primary antibodies and were incubated for 2h at RT or ON at 4°C.
The coverslips were washed 2x10min in PBS and put on 40μl of secondary antibody. They were stained in the dark for 1h at 37°C. After another 2x10min washing in PBS (in the dark) the coverslips were mounted with 6μl mounting media containing DAPI and fixed with nail polish on glass slides.
The pictures were made by fluorescent microscopy on a Leica DMI6000B automated inverted research microscope system (Leica Microsystems®). Composite pictures were generated by Adobe® Photoshop software.

3.3.3 Scratch wound healing migration assay

DU145 or PC3 cells were seeded into 6 wells (0.2×10^6 cells/well) and transfected with siRNA as indicated. After 24h the cells were trypsinised and 400'000 cells were pooled into one well. After 24-36 h when cells reached confluency, identical scratches were made in parallel wells using a 1000 µl plastic pipette tip. Non-adherent cells were removed by two washes. The closure of the scratch was analyzed under the microscope and images were captured at 0, 12, 24, and 36 h after incubation. Photographs were made with a Leica DMI6000B automated inverted research microscope system (Leica Microsystems®) at the indicated time points. The size of the uncovered areas was measured with Adobe Photoshop software and converted into percentages. For analysis of the migration potential, mean values of three independent experiments were analyzed. Mean value ± SE was calculated and plotted into graphs with GraphPad Prism 5 software (GraphPad Software, Inc.).

3.3.4 Trypan-blue exclusion assay and proliferation assay

Cells were seeded at 0.2 × 10^6 cells/well in 6-well dishes 8-12 h prior to initiation of treatment and then incubated in the presence of PBS, DMSO (mock-treated), IFNy (200U/ml) or Docetaxel (0.5-1 nM) for 24 h.
The medium was put in a tube and centrifuged for 5min with 5000rpm. The supernatant was discarded and the cells were resuspended in 0.5ml sterile PBS. The adherent cells were detached from the 6-wells by PBS (1ml) and mixed with the cells from the supernatant.
Each probe was shaken well and 10µl were mixed with 10µl of 0.4% trypan-blue. After 1min incubation the probe was counted in an improved Neubauer counting chamber. Each probe was counted 4 times.
Relative cell viability/proliferation and cell numbers are presented as means from three independent experiments performed in triplicate ± SE. All data were analyzed with Excel (Microsoft® Inc.) and GraphPad Prism 5 software. Analyzed data were plotted into graphs using the GraphPad Prism 5 software (GraphPad Software, Inc.).

3.3.5 Gene expression analyses

Total RNA was isolated using Tri-Reagent (MRC, Inc) according to manufacturers protocols. RNA was subsequently reverse-transcribed using the 'High-capacity cDNA reverse transcription' kit (Applied Biosystems) according to manufacturers protocols. qPCR was performed using the Rotor-Gene 3000 (Corbett Life Science, now Qiagen®) and SYBR Green kit (Bioline®) according to manufacturers' protocols using the primers listed in Table 3. Mean value ± SE was calculated and blotted into graphs with GraphPad Prism 5 software (GraphPad Software).

3.3.6 Statistical analyses

The results of three independently performed experiments, each performed in triplicates, were summarized as mean and SE. Statistical evaluations (comparisons between control and treated groups) were established by Student's t-test for unpaired data (for two comparisons). P values <0.05 were considered statistically significant. All statistical evaluations were performed with GraphPad Prism 5 software (GraphPad Software, Inc.).

4 Results

4.1 DTX3L, ARTD8 and ARTD9 are overexpressed in PC3 and DU145 cells

In order to test whether DTX3L and ARTD9 are also overexpressed in prostate cancer cell, expression levels of DTX3L and ARTD9 were analyzed in different prostate cancer cell lines. The following cell lines were used:

- PC3 and DU145 : p53-negative or p53-mutant, androgen-refractory and highly tumorigenic cell lines [75-79] .
- LNCaP: p53-positive androgen sensitive, JAK-1 negative and poorly tumorigenic cell line [77-84].
- HPE and RWPE1: normal prostate luminal epithelial cell lines.

Indeed, the immunoblot analyses showed an overexpression of both DTX3L and ARTD9 in PC3 and DU145 cells compared to the normal epithelial prostate cells. In the LNCaP cells there was only a slight overexpression of DTX3L detectable (Figure 6). The DLBCL cell line SUDHL7 was used as positive control.
ARTD8 was expressed in PC3 and DU145 cell lines, too.

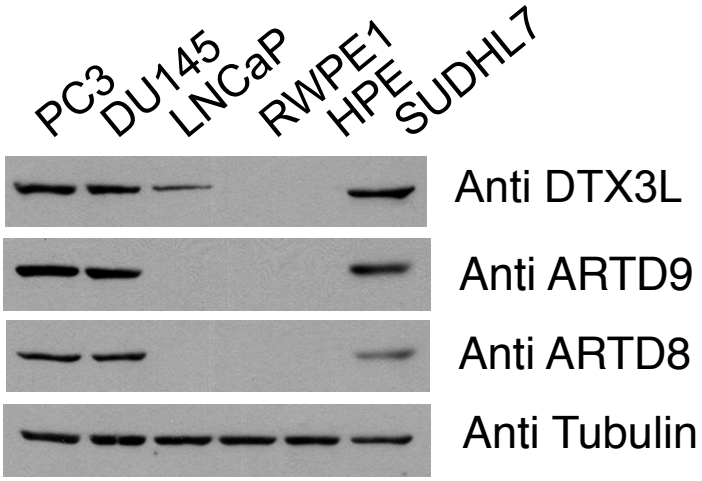

Figure 6: DTX3L, ARTD9 and ARTD8 are expressed in different prostate cancer cell lines. Immunoblot analyses of three different prostate cancer cell lines (PC3, DU145, LNCaP), two immortalized normal prostate luminal epithelial cell lines (RWPE1 and HPE) and SUDHL7 (SUDHL7 was used as a positive control). ARTD9, ARTD8 and DTX3L are expressed in PC3 and DU145 cells, but not in RWPE1 or HPE cells. In LNCaP cells only DTX3L is expressed.

To investigate the cellular localization of these three proteins, immunofluorescence analyses were performed. Because it is known that DTX3L and ARTD9 are induced upon the stimulation of IFNγ [60], these analyses were done with or without the stimulation of the cells with 200U/ml IFNγ for 2 hours. Both DTX3L as well as ARTD9 are mainly localized in the cytoplasm (Figure 7A and B) whereas only small subfractions show nuclear localization. They stay mainly in the cytoplasm after the stimulation with IFNγ.
After IFNγ stimulation an enhanced signal was detected, especially in DTX3L.
ARTD8 is evenly distributed in the nucleus and cytoplasm in these cells.
The same results were seen in DU145 cells.

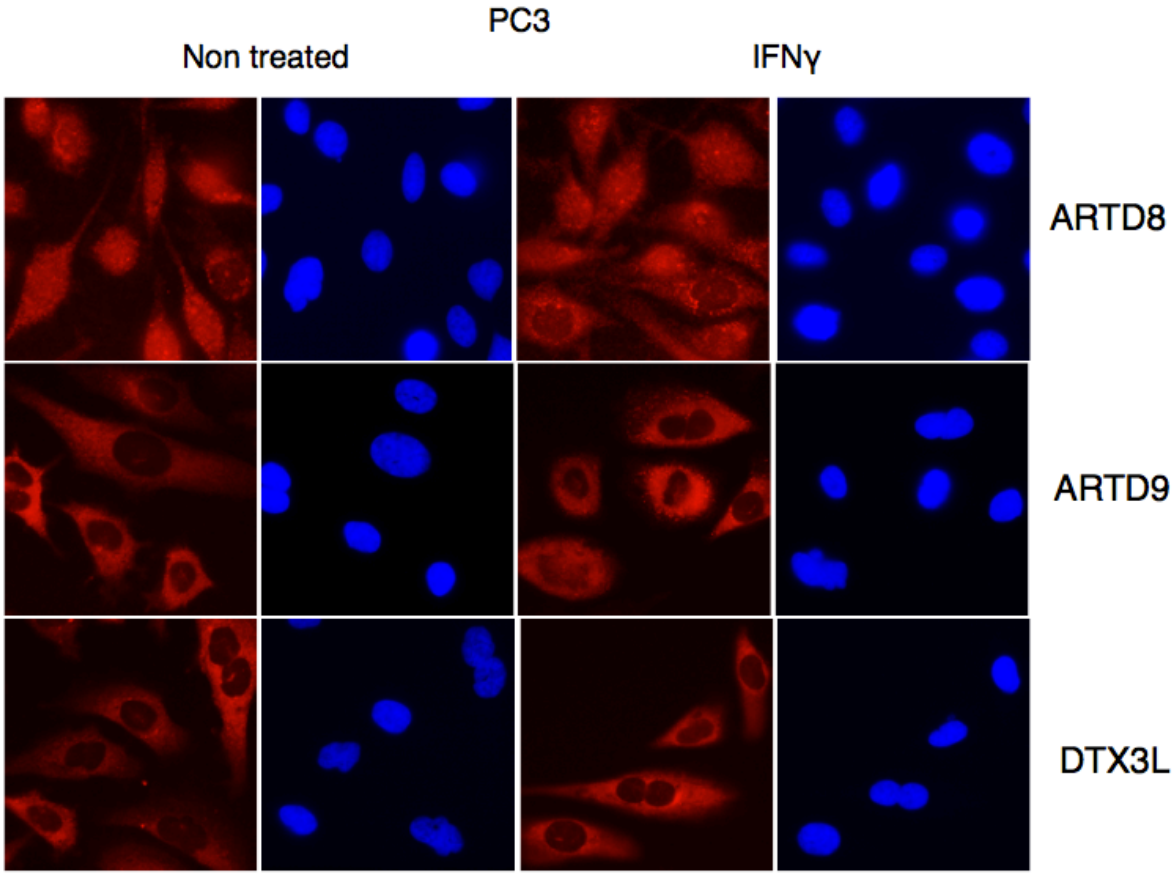

Figure 7: Subcellular localization of ARTD8, ARTD9 and DTX3L in PC3 cells.
Immunofluorescence microscopy analyses show the localization of ARTD9 and DTX3L in PC3 cells without and with a stimulation of 200U/ml IFNγ over 2 hours. Both proteins stay mainly in the cytoplasm, independently of INFγ treatment. ARTD8 is evenly distributed in the nucleus and cytoplasm.

DTX3L is a nucleocytoplasmic shuttling protein and complex formation between DTX3L and ARTD9 in the nucleus has been suggested to facilitate the nuclear export of ARTD9 by DTX3L [60]. However, the subsequent siRNA-knockdown experiments revealed that endogenous DTX3L does not facilitate the nuclear export of ARTD9 in PC3 cells. ARTD9 is mainly localized in the cytoplasm in both siMock (Figure 8A) and siDTX3L PC3 cells (Fig. 8B). The same pattern was observed for DTX3L in siMock and siARTD9 PC3 cells (Fig. 8C), strongly indicating that the nuclear shuttling of ARTD9 is mainly regulated by other factors, and thus, the previously observed nuclear export of ectopically overexpressed fluorescent protein-tagged-ARTD9 by ectopically overexpressed fluorescent protein-tagged-DTX3L [60] most likely represents a mechanism highly specific to cell type and stimuli.

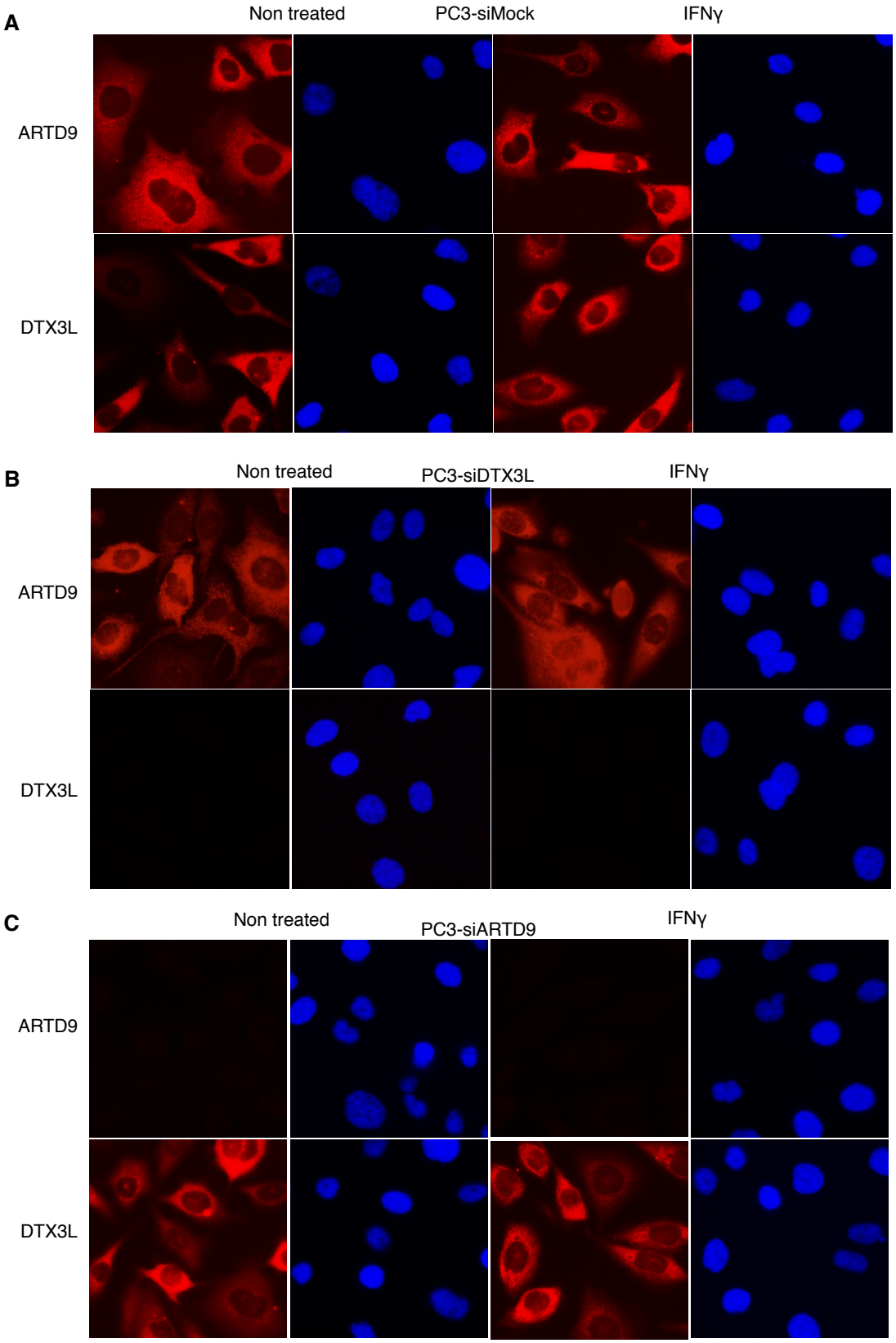

Figure 8: DTX3L and ARTD9 do not cross-regulate their subcellular localization. A: Immunofluorescence microscopy analysis with siMock PC3 cells of DTX3L and ARTD9, ± IFNγ. Both proteins stay mainly in the cytoplasm. B: Immunofluorescence microscopy analysis with siDTX3L PC3 cells of DTX3L and ARTD9, ± IFNγ. ARTD9 stays mainly in the cytoplasm, unaffected of the absence of DTX3L. C: Immunofluorescence microscopy analysis with siARTD9 PC3 cells of DTX3L and ARTD9, ± IFNγ. DTX3L stays mainly in the cytoplasm, unaffected of the absence of ARTD9.

4.2 Knockdown of DTX3L, ARTD9 and ARTD8 in PC3 and DU145 cells

In order to study the influences of DTX3L, ARTD9 and ARTD8, they had to be silenced successfully first. To define the silencing efficiency immunoblot analyses were done. The cells were also stimulated with IFNγ to show that the silencing is still working under stimulated conditions.
The immunoblot analysis showed not only a silencing of the targeted protein, but also an influence between DTX3L and ARTD9 (Figure 9A and B). If one of them was silenced the protein level of the other one was always also reduced. This could reflect the special condition of the bidirectional promotor which these two proteins share [60]. DTX3L and ARTD9 not only share a bidirectional promoter but also regulate each other on the level of transcription. It has been previously shown that ARTD9 and DTX3L can bind to their own promoter [72].
The silencing efficiency was also tested on the RNA level, by performing quantitative RT-PCR (Figure 9F).
The silencing efficiency of DTX3l, ARTD8 and ARTD9 is shown in Figure 9C.
DTX3L and ARTD9 were also successfully silenced in DU145 cells (Figure 9D,E).

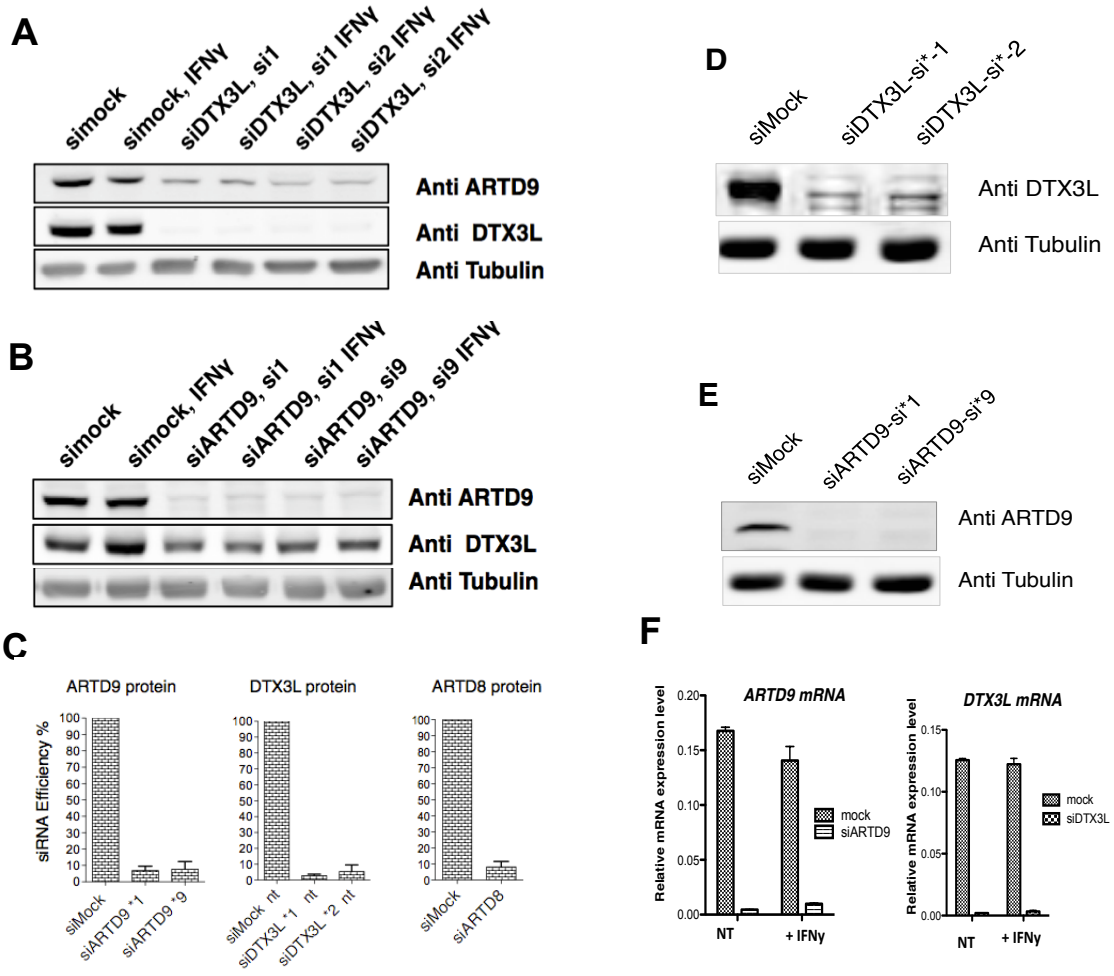

Figure 9: Silencing DTX3L, ARTD9 and ARTD8 in PC3 and DU145 cells. A: Silencing of DTX3L in PC3 cells with two different siRNAs and the treatment with IFNγ. The protein level of ARTD9 is reduced as well. B: Silencing of ARTD9 in PC3 cells with two different siRNAs and the treatment with IFNγ. The protein level of DTX3L is reduced as well. C: Relative silencing efficancy of ARDT9, DTX3L and ARTD8 in PC3 cells. D: Immunoblot analysis of siDTX3L and siMock DU145 cells. E: Immunoblot analysis of siARTD9 and siMock DU145 cells. F: Relative mRNA expression levels of siARTD9 and siDTX3L PC3 cells ± IFNγ compared to siMock ± IFNγ.

4.3 DTX3L, ARTD9 and ARTD8 mediate proliferation in PC3 cells

It has been recently shown that ARTD9 is required for proliferation of diffuse large B cell lymphoma (DLBCL) cells. Moreover it has been shown that ARTD8 mediates proliferation and survival of B cells [55, 54, 61]. DTX3L, ARTD8 and ARTD9 might therefore also play such a role in prostate cancer cells.
In order to investigate this hypothesis, PC3 cells were silenced for DTX3L, ARTD8 or ARTD9 using siRNA. IFNγ was used as an additional extrinsic inhibitory stimulus. It is known that IFNγ inhibits proliferation in p53-negative, androgen-refractory prostate cancer cells [85, 86], such as PC3. The results showed a significant decrease of the proliferation in PC3 cells after the stimulation with INFγ (Figure 10A-C). Indeed, silencing of both DTX3L and ARTD9 strongly inhibits cell proliferation of PC3 cells while depletion of ARTD8 has only a minor effect. As expected from a previous study in DLBCL, the negative effect of IFNγ on the proliferation was significantly higher in cells depleted of DTX3L or ARTD9, indicating that DTX3L acts together with ARTD9 in IFNγ/STAT-1-signalling pathways. On the other hand, the inhibition of proliferation by IFNγ was not further enhanced in ARTD8 depleted cells, strongly indicating that ARTD8 does not act in IFNγ/STAT-1-signalling pathways.

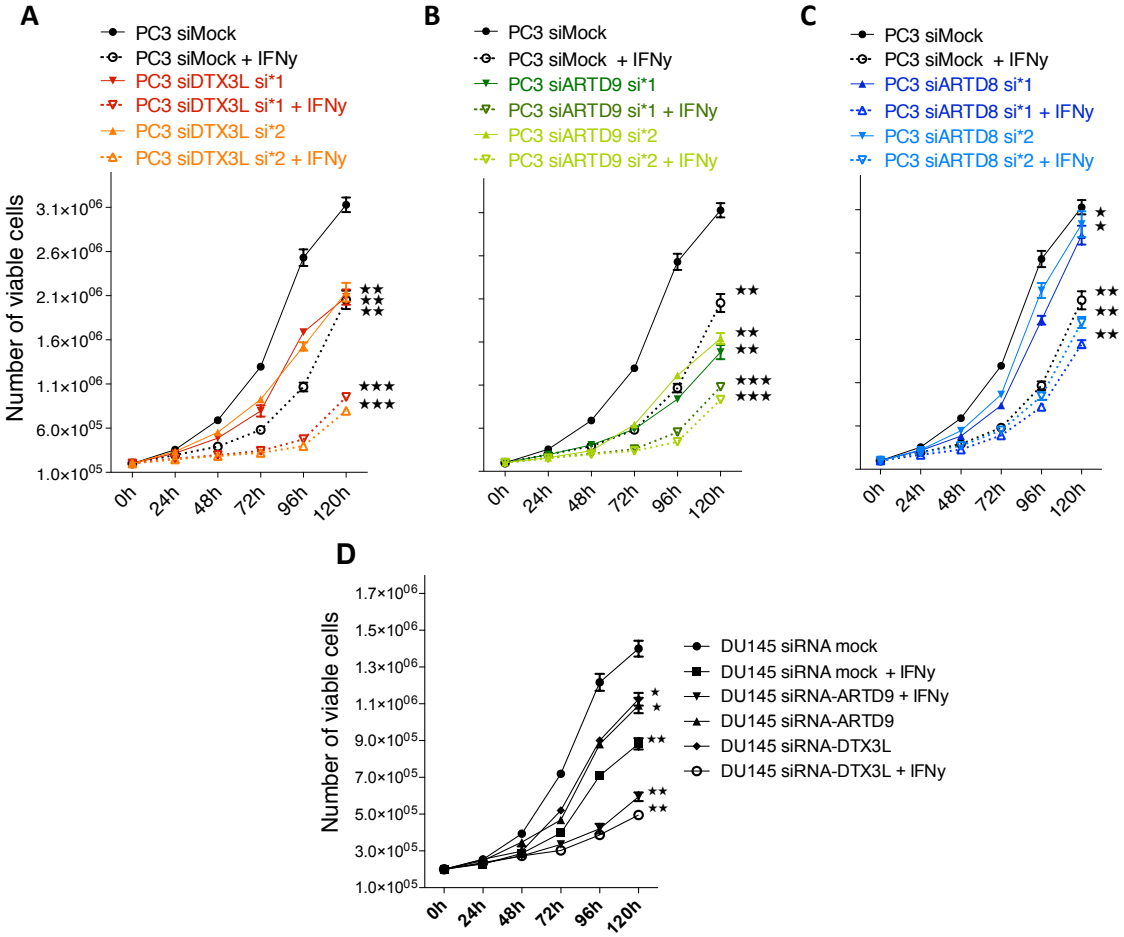

Figure 10: DTX3L and ARTD9 inhibit proliferation in PC3 and DU145 cells. A: Proliferation assay with PC3 cells, siMock, siDTX3L and treated ± IFNγ. There is a significant inhibition of the proliferation in siDTX3L cells. B: Proliferation assay with PC3 cells, siMock, siARTD9 and treated ± IFNγ. There is a significant inhibition of the proliferation in siARTD9 cells C: Proliferation assay with PC3 cells, siMock, siARTD8 and treated ±IFNγ. There is a significant inhibition of the proliferation in siARTD8 cells as well, but less pronounced compared to siDTX3L or siARTD9 cells. D: Proliferation assay with DU145 cells, siMock, siDTX3L or siARTD9 ± IFNγ. There is a significant inhibition of proliferation in siDTX3L and siARTD9 cells. Statistical analysis was performed using Student's t-test, comparing each sample with siMock. *P<0.05, **P<0.001, ***P<0.0001. Error bars indicate S.D.

The same observations could be seen in another cell line of prostate cancer, DU145. The silencing of DTX3L or ARTD9 resulted in a significantly decreased proliferation in DU145 cells (Figure 10D). Again the IFNγ treatment was used as a positive control of the inhibition of proliferation. Like in PC3 cells the combination of the silencing of either DTX3L or ARTD9 and IFNγ treatment inhibited the proliferation most effectively.

4.4 Crosstalk between DTX3L, ARTD9 and ARTD8 also mediates survival and chemo-resistance in PC3 cells

Since it is known that ARTD9 also mediates survival and chemo-resistance in diffuse large B-cell lymphomas (DLBCL) cells [72], survival assays were performed in PC3 cells with siMock, siDTX3L, siARTD9 and siARTD8 cells.
Cells were kept in a control medium containing the highest amount of solvents used (CT), or kept in normal medium (NT), treated with IFNγ (IFN), or Docetaxel (DT) for 48 hours. The trypan exclusion assays showed a significant increase of the sensitivity towards IFNγ in siDTX3L and siARTD9 cells (Figure11A). It was shown that the pro-apoptotic effect of IFNγ, which is absent in siMock PC3 cells, returns in the siDTX3L and siARTD9 cells. Conversely no significant difference could be observed in the siARTD8 cells upon treatment with IFNγ, when compared to siMock cells.
All three different silenced cells showed a significant higher sensitivity towards the chemotherapeutic drug Docetaxel. In addition, the combinational treatment with IFNγ and Docetaxel had a significantly stronger effect in the silenced cells compared to the siMock cells.
To investigate whether there is a crosstalk between DTX3L, ARTD9 and ARTD8, the same survival assays were performed with siARTD9/siDTX3L, siARTD8/siDTX3L and siARTD9/siARTD8 double knockdown cells. The concentration of Docetaxel was reduced from 1nM to 0.5nM in order to analyze the difference between single- and double-knockdown more easily.
These trypan exclusion assays showed additive or synergistic effects in the sensitivity toward Docetaxel treatment only when DTX3L or ARTD9 were silenced together with ARTD8 (Figure 11B). There was no significant effect in the double-knockdown of siDTX3L/siARTD9 compared to their single-knockdowns.

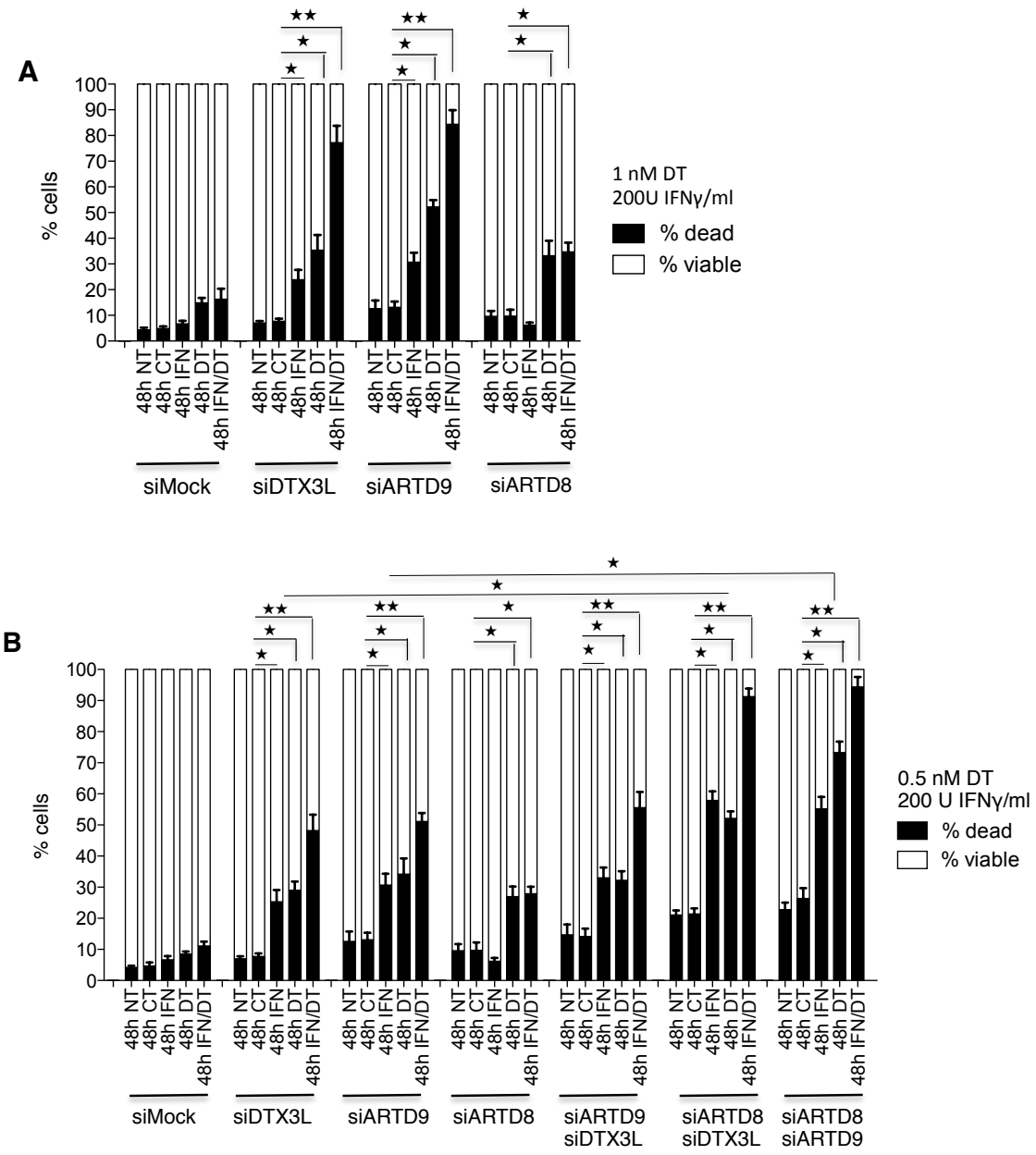

Figure 11: DTX3L, ARTD9 and ARTD8 mediate survival and chemo-resistance in PC3 cells. A: Trypan exclusion assay with siMock, siDTX3L, siARTD9 and siARTD8 PC3 cells. In siDTX3L, siARTD9 and, in a less pronounced way, in siARTD8 cells the treatment shows a stronger effect compared to siMock cells. B: Trypan exclusion assay with siMock, siDTX3L, siARTD9, siARTD8 and also the double knockdowns siARTD9/siDTX3L, siARTD8/siDTX3L, siARTD8/siARTD9. There is no addidirve effect of the double knockdown with siARTD9/siDTX3L. The double knockdowns of siARTD8/siDTX3L and siARTD8/siARTD9 show an additive effect. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001, ***P<0.0001. Error bars indicate S.D.

4.5 DTX3L and ARTD9 mediate proliferation, survival and chemo-resistance in PC3 cells in a STAT-1-dependent manner

Due to our observation that ARTD9-mediated proliferation of DLBCL is STAT-1 dependent [72], the effects of STAT-1 in PC3 cells were analyzed.
Indeed, single knockdowns of siSTAT-1 and siDTX3L and the double-knockdown siSTAT-1/siDTX3L showed the same effect (Figure 12A and B). Similar to the

situation in DLBCL cells, the detrimental effects of IFNγ treatment on the proliferation of siDTX3L or siARTD9 cells could be rescued upon depletion of STAT-1, because the IFNγ was no more able to activate the STAT-1-dependent antiproliferative pathways. On the other hand the silencing of both ARTD8 and STAT-1 showed an additive effect when compared to the single knockdown cells (Figure 12C) again indicating that STAT-1 and ARTD8 synergistically act in proliferation but not in the same signaling pathways.

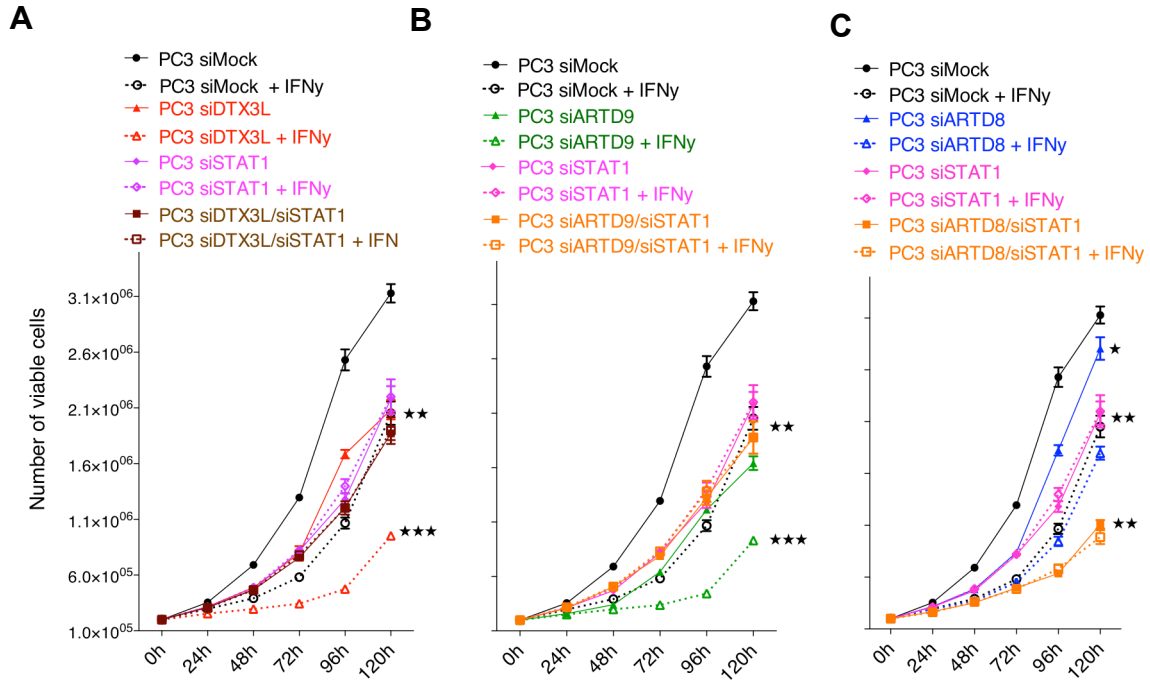

Figure 12: Proliferative effects mediated by DTX3L and ARTD9 are dependent of STAT-1. A: The cells were silenced either for DTX3L, STAT-1 or both, ± IFNγ. The double-knockdowns have the same effect as the single kockdowns. INFγ loses its effect if STAT-1 is silenced. B: The cells were silenced either for ARTD9, STAT-1 or both ± IFNγ. Again the double-knockdowns have the same effect as the single kockdowns. INFγ loses its effect if STAT-1 is silenced. C: The cells were silenced either for ARTD8, STAT-1 or both ± IFNγ. The knockdown of ARTD8 has less effect on proliferation then the double-knockdown of ARTD8/STAT-1. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001, ***P<0.0001, comparing each sample with siMock. Error bars indicate S.D.

The survival assays show a higher mortality rate in STAT-1 silenced cells compared to the siMock cells (Figure 13). As expected from the proliferation assays silencing of STAT-1 in cells depleted of DTX3L or ARTD9 showed a rescue effect in these cells in absence and presence of IFNγ, when compared to the single knockdown cells (Figure 13) (siDTX3L is not shown in the figure for the clarity of the figure. The significances were the same as for ARTD9).
The double-knockdown of STAT-1 and ARTD8 had a further negative effect on the cells viability. Therefore the effects of ARTD8 and STAT-1 are additive or synergistically.

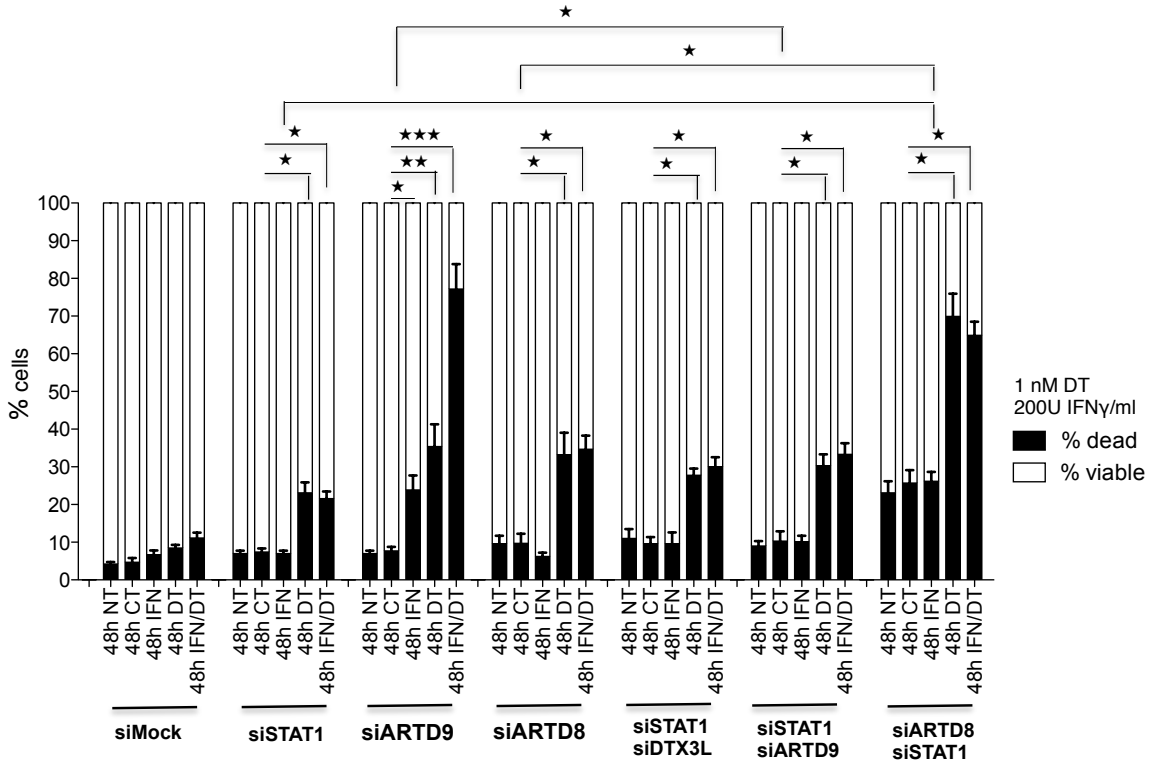

Figure 13: DTX3L and ARTD9 mediated survival and chemoresistance is dependent on STAT-1. Single knockdown of STAT-1, ARTD9, ARTD8 and the correspondent double-knockdowns with STAT-1 in PC3 cells. The double-knockdowns of STAT-1 with DTX3L or ARTD9 show a rescue effect compared to the knockdowns of DTX3L or ARTD9. The double-kockdown of STAT-1/ARTD8 has a stronger effect compared to the single knockdowns of STAT-1 or ARTD8. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001, ***P<0.0001. Error bars indicate S.D.

4.6 pSTAT-1-S727 is constitutively active in PC3 and DU145 cells

To learn more about the role of STAT-1 in prostate cancer cells, immunofluorescence microscopy analyses were done. PC3 cells were tested for STAT-1, pSTAT-1-Y701 and pSTAT-1-S727. Also the influence of 200U IFNγ for 2 hours was analyzed (Figure 14).
The STAT-1 pathway is well studied [17, 20, 23, 24] and therefore it is known that STAT-1 is normally activated by IFNγ and then gets phosphorylated on either tyrosine 701 or serine 727 to finally form dimers and translocate into the nucleus. There they display their effect in activating the transcription of specific genes.
The immunofluorescence microscopy analyses in PC3 cells showed this behavior only for pSTAT-1-S701 (Figure 14), it was only slightly detectable without any stimulation and was located mainly in the cytoplasm. After the stimulation with INFγ there was a strong increase of the signal and the protein was localized mainly in the nucleus. pSTAT-1-Y727 on the other hand stayed in the nucleus independently of the stimulation with INFγ, only the intensity of the signal was slightly stronger (Figure 14). The same results were seen in DU145 cells.

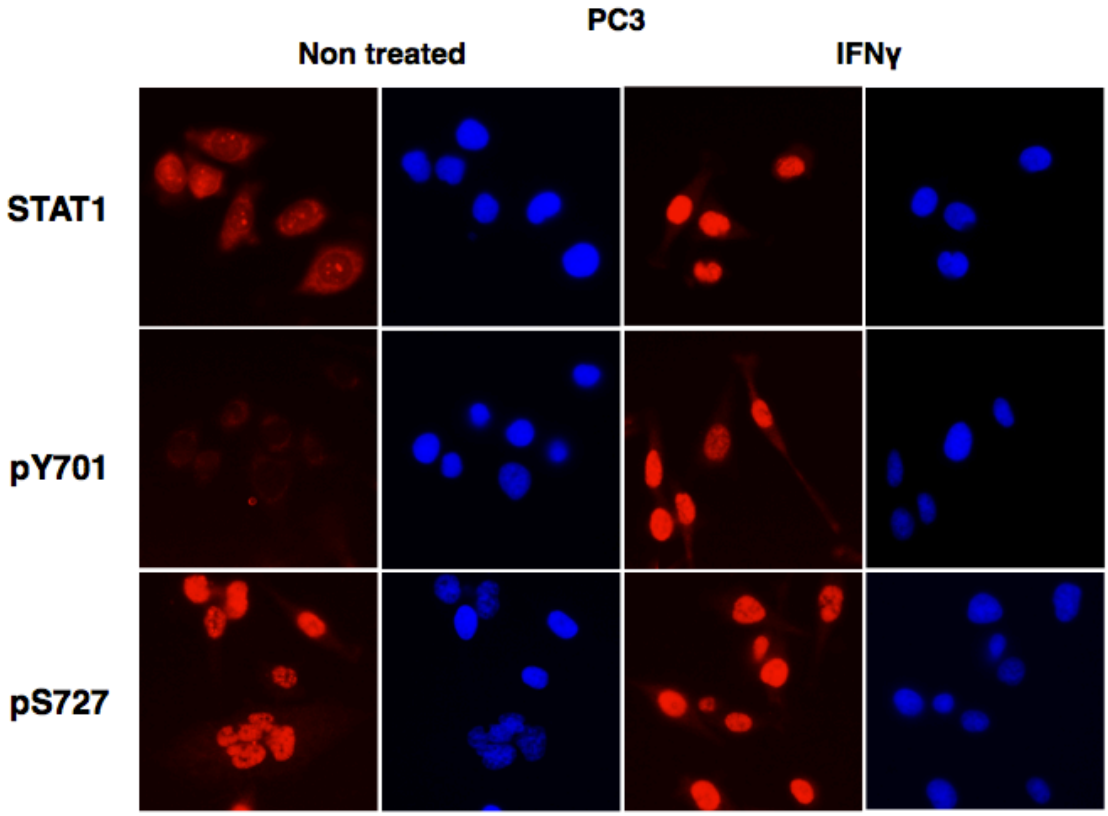

Figure 14: pSTAT-1-S727 is constitutively active in PC3 cells. Immunofluorescence microscopy analyses of PC3 cells ± IFNγ of STAT-1, pSTAT-1-Y701, pSTAT-1-S727. Y701 was only activated after the stimulation with IFNγ. S727 was activated independently of IFNγ and stayed in the nucleus.

4.7 DTX3L and ARTD9 repress IRF1 expression in prostate cancer cells

In order to investigate the role of IRF1 in cell proliferation and survival and to test whether the negative effects mediated by Stat1 are based on IRF1 a closer look at the mayor downstream targets of the STAT-1 pathway was indicated. Immunoblot analyses in siMock, siDTX3L and siARTD9 PC3 cells were done. Although IRF7 has been suggested to be a target of ARTD9 [60], there was no effect on the protein levels of IRF7 in siDTX3L or siARTD9 PC3 cells (Figure 15A and B).
On the other hand, the immunoblot analysis showed an increased protein level of IRF1 in siDTX3L and siARTD9 cells. The quantification of these blots showed a significant increase of IRF1 in the silenced cells (Figure 15C and D). Therefore DTX3L and ARTD9 repress the tumor suppressor IRF1.
The same result could be seen on the mRNA levels, analyzed by qPCR (Figure 15E).

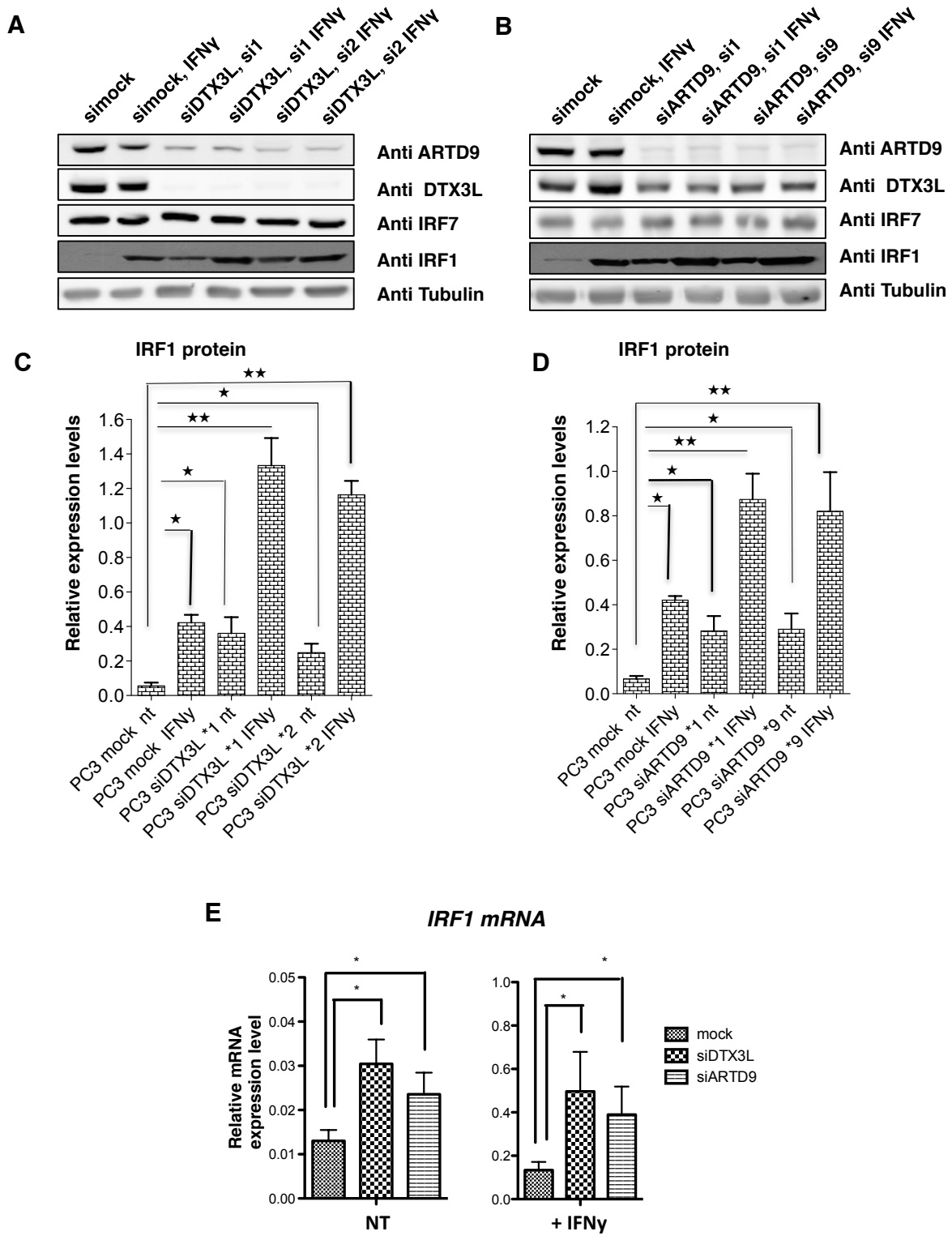

Figure 15: DTX3L and ARTD9 suppress IRF1. A and B: Immunoblot analyses with siMock, siDTX3L and siARTD9 PC3 cells ± 200U IFNγ for 2h. The protein level of IRF1 was induced upon IFNγ and increased in the silenced cells. C and D: Quantification of A and B, the protein level of IRF1 is significantly enhanced after silencing DTX3L or ARTD9. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001. Error bars represent S.D. E: Quantified qPCR data of siMock, siDTX3L and siARTD9 PC3 cells ± IFNγ, mRNA levels of IRF1 are significantly enhanced in siDTX3L and siARTD9 cells compared to siMock cells. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001. Error bars represent S.D.

In order to learn more about the functions of IRF1 cell proliferation and survival was investigated in PC3 cells depleted of IRF1. The immunoblot analysis for the quantification of siRNA efficiency was done with cells treated with IFNγ, because the IRF1 protein levels in unstimulated cells were not detectable (Figure 16A). The silencing was also controlled on the mRNA level, performing qPCR (Figure 16B).

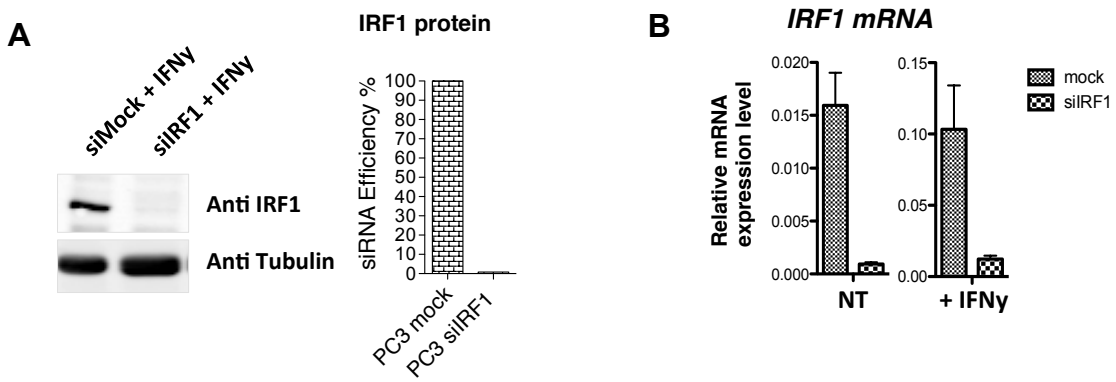

Figure 16: Silencing IRF1 in PC3 cells. A: Immunoblot analyses of siMock and siIRF1 PC3 cells, stimulated with IFNγ and its quantification. B: qPCR analyses of siMock and siIRF1 PC3 cells. Error bars indicate S.D.

In order to control if the STAT-1 dependent influences of DTX3L and ARTD9 in PC3 cells are based on IRF1, proliferation and survival assays were performed. Only the proliferation assay showed some influence of IRF1 (Figure 17).
For the proliferation assay siMock and siIRF1 PC3 cells were used. Again part of the cells were kept under the stimulation of IFNγ as a positive control. There was an increased proliferation of the siIRF cells compared to the siMock cells. Also the IFNγ treatment had less effect in the siIRF1 cells.

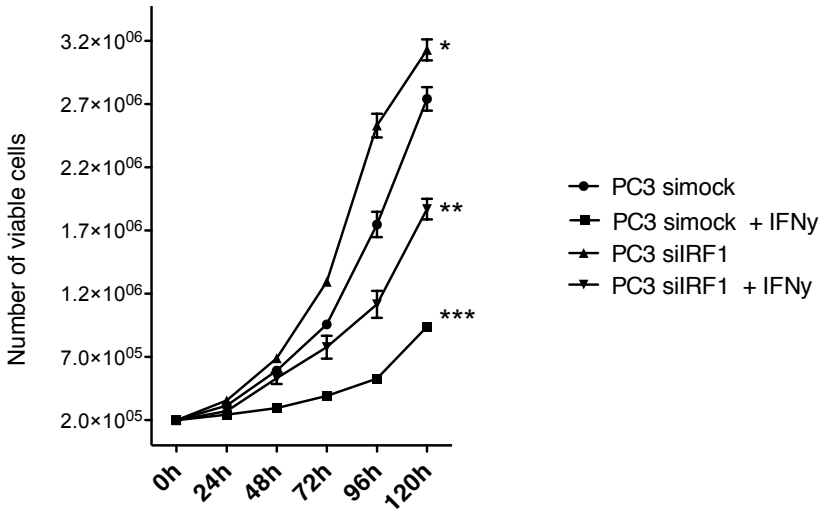

Figure 17: IRF1 inhibits proliferation in PC3 cells. Proliferation assay performed with siMock and siIRF1 PC3 cells ± INFγ. siIRF1 cells show an enhanced proliferation compared to siMock cells. Statistical analysis was performed using Student's t-test, comparing each sample with siMock. *P<0.05, **P<0.001, ***P<0.0001. Error bars represent S.D.

4.8 DTX3L mediates cell migration of PCa cells in a STAT-1 and STAT-3 dependent manner

Studies showed that ARTD9 might be associated with lymphocyte migration [52, 60]. ARTD9 was ectopically enhanced in an ARTD9- and DTX3L-negative DLBCL cell line. The migration in these cells was strongly enhanced compared to the control cells [60]. In order to test whether endogenous ARTD9 or DTX3L might also be associated with migration in prostate cancer cells, migration assays were performed. Therefore siMock, siDTX3L and siARTD9 cells of the cell lines PC3 and DU145 were analyzed using the classical scratch migration assay for adherent cells.
The quantification of the assays showed a significantly reduced migration in siDTX3L cells in both cell lines but no influence of ARTD9 on migration could be observed (Figure18A-D). Also siARTD8 cells were used, but no effect could be seen.

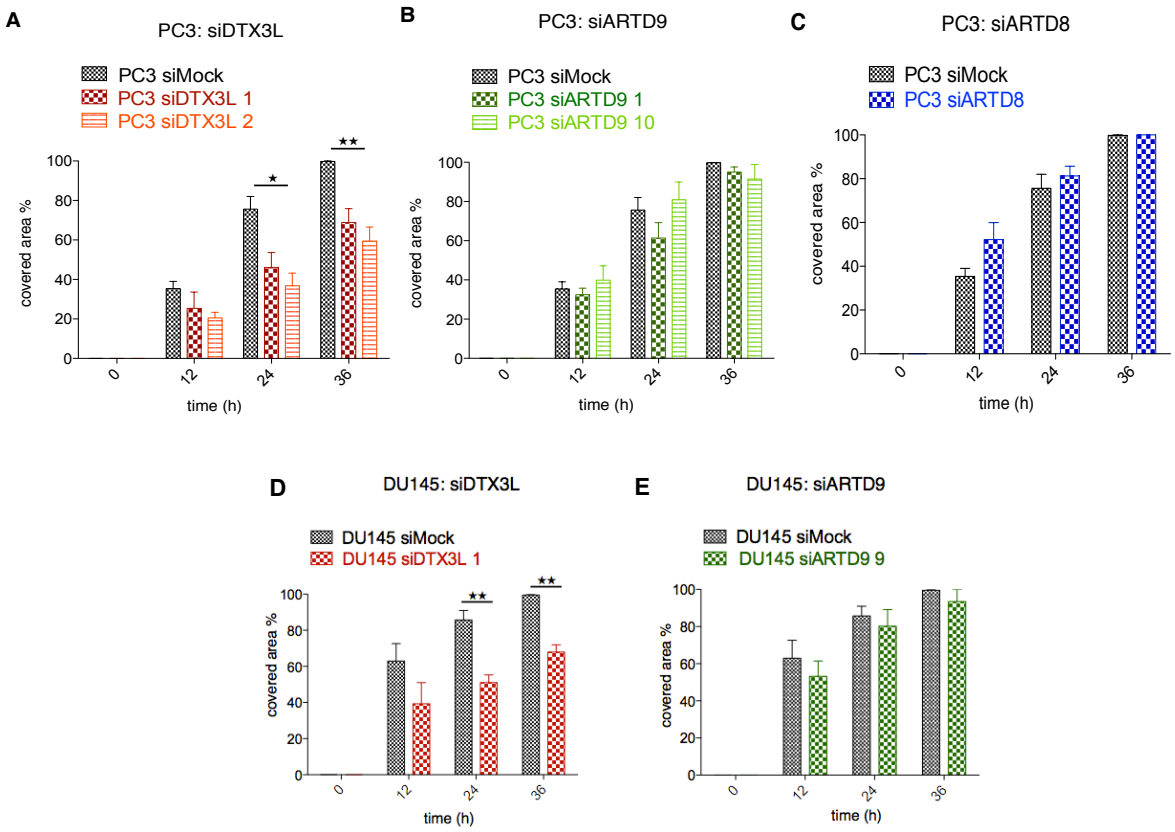

Figure 18: Influence of DTX3L, ARTD9 and ARTD8 on migration in PC3 and DU145 cells. A-C: siDTX3L, si ARTD9 and siARTD8 cells compared to siMock cells in PC3 cells. Only the siDTX3L cells showed a significantly decreased migration compared to siMock cells. D and E: siDTX3L and siARTD9 cells compared to siMock cells in DU145 cells. Again only siDTX3L cells showed a significantly reduced migration compared to siMock cells. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001, ***P<0.0001. Error bars represent S.D.

Recent studies showed that STAT-1 plays a role in migration of prostate cancer cells [87, 88]. Therefore STAT-1 and STAT-3, which is known to mediate tumor migration, invasion and metastases [26, 46], were analyzed in migration assays.

These experiments showed also a significantly reduced capability of migration in siSTAT-1 as well as siSTAT-3 cells when compared to the siMock cells (Figure 19A-D). This effect was seen in both, PC3 and DU145 cells.

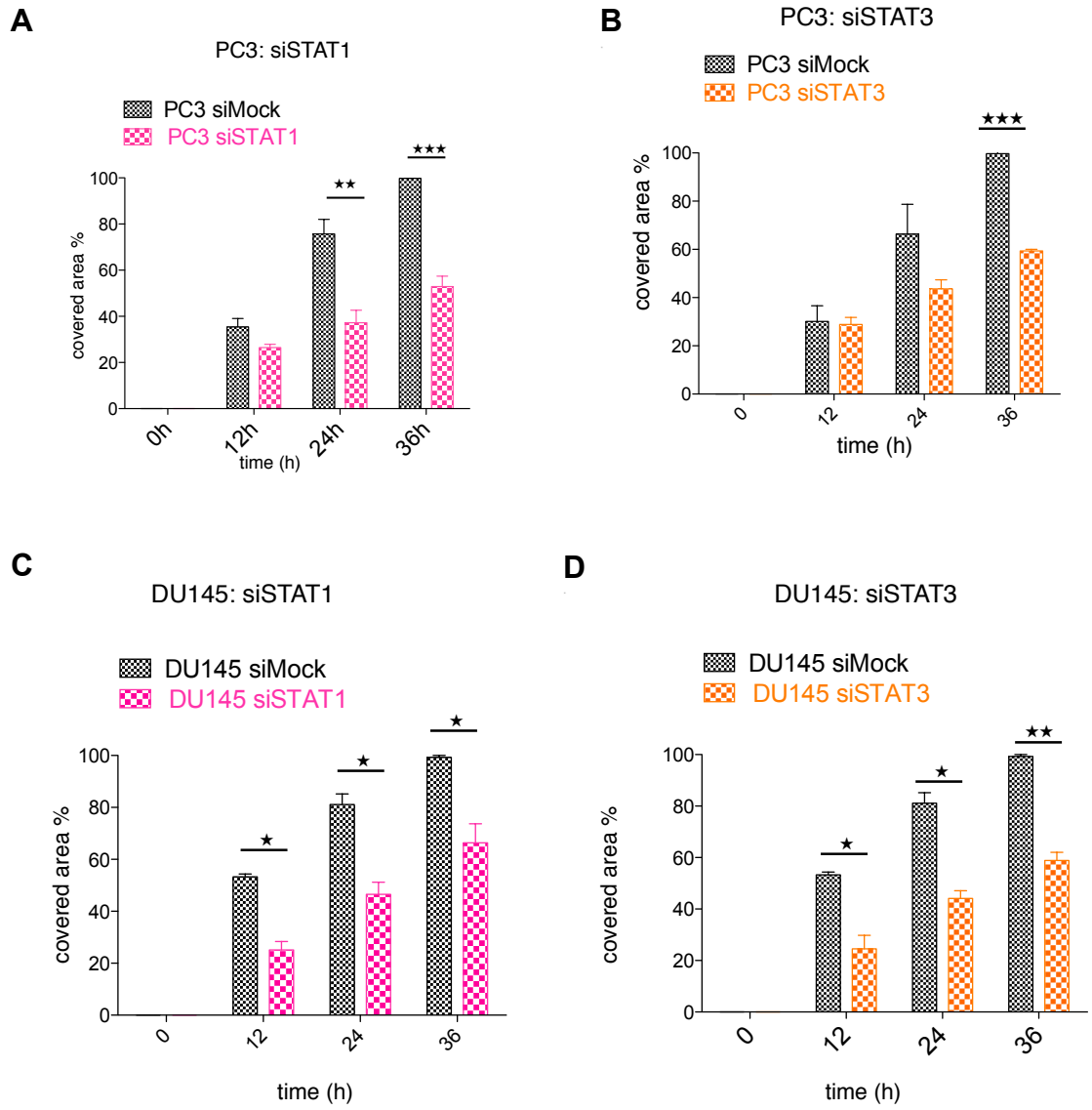

Figure 19: Influence of STAT-1 and STAT-3 on migration in PC3 and DU145 cells. A and B: siSTAT-1 cells showed a significantly decreased migration in PC3 and DU145 cells compared to siMock cells. C and D: siSTAT-3 cells showed a significantly decreased migration in PC3 and DU145 cells compared to siMock cells. Statistical analysis was performed using Student's t-test. *P<0.05, **P<0.001, ***P<0.0001. Error bars represent S.D.

In a next step the migration assays were performed with siDTX3L/siSTAT-1 and siDTX3L/siSTAT-3 double-knockdown cells. No further inhibition of migration could be observed in the double-knockdowns. Even triple-knockdowns of siDTX3L, siSTAT-1 and siSTAT-3 had no further negative effect on migration.
To investigate whether the effect of STAT-1 was again dependent on IRF1 or not, the migration assay was also performed with siIRF1 PC3 cells. No significant influence of IRF1 on migration could be detected.

5 Discussion

DTX3L, originally discovered in B-cell lymphomas, has since been detected in different types of cancer like cervical carcinomas [89] or prostate carcinoma. Therefore the importance of DTX3L in tumorigenesis seems to be greater than originally expected.

5.1 Regulation of DTX3L and ARTD9 in prostate cancer cells

Here it is shown that DTX3L, ARTD8 and ARTD9 are overexpressed in the highly tumorigenic metastatic prostate cancer (mPCa) cell lines, PC3 and DU145. Conversely, ARTD8 and ARTD9 are not expressed in LNCaP cells and DTX3L is only slightly expressed in this cell line.
Both mPCa cell lines PC3 and DU145 have a high metastatic potential [77, 78, 90, 91] and are highly invasive compared to the poorly metastatic LNCaP cells [91-94]. Contrary to the LNCaP cell line, the PC3 and DU145 cell lines have been previously described as having enhanced basal levels of active STAT-1 and expressing high levels of IL6 [95, 98]. Both, PC3 and DU145 cell lines, have also been recently described as having an autocrine IL6 loop while LNCaP does not have any detectable IL6 secretion [95].
There is also first evidence that expression of ARTD9 and DTX3L is also induced by IL6 and strongly associated with an autocrine IL6 signaling loop in prostate cancer cells [95]. IL6 mainly activates STAT-3, but under certain conditions, it can also activate STAT-1 [26, 28, 96, 97]. Consequently the results shown here strongly suggest that the constitutive overexpression of DTX3L and ARTD9 in PC3 and DU145 cells is most likely mediated through an IL6/JAK1-STAT-1:STAT-3-signaling pathway (Figure 20).
The fact that DTX3L and ARTD9 protein levels are up-regulated after the stimulation with IFNγ, indicates a further up-regulation through an IFNγ/JAK1-STAT-1:STAT-1 mediated signaling pathway (Figure 20).
On the other hand, DTX3L is still expressed in the JAK1 and IL6-negative prostate carcinoma cell line LNCaP, though to a much lesser extent. That fact demonstrates the possibility of a cell type-specific regulation of DTX3L which is independent of ARTD9, IFNγ/STAT-1 and IL6/STAT-3 signaling (Figure 20).
The knockdown experiments with siDTX3L and siARTD9 cells also show the influence between these two proteins. If one is silenced, the protein level of the other is also remarkably reduced. Although the immunofluorescence microscopy analyses showed, that DTX3L and ARTD9 are mainly localized in the cytoplasm and only small subfractions have nuclear localization, their possible influence in the nucleus is not ruled out. Aguiar et. al. [52] showed clearly the nuclear localization of ARTD9 in B-cells. Therefore DTX3L and ARTD9 could tightly regulate each other through a positive feedback loop on the level of transcription.

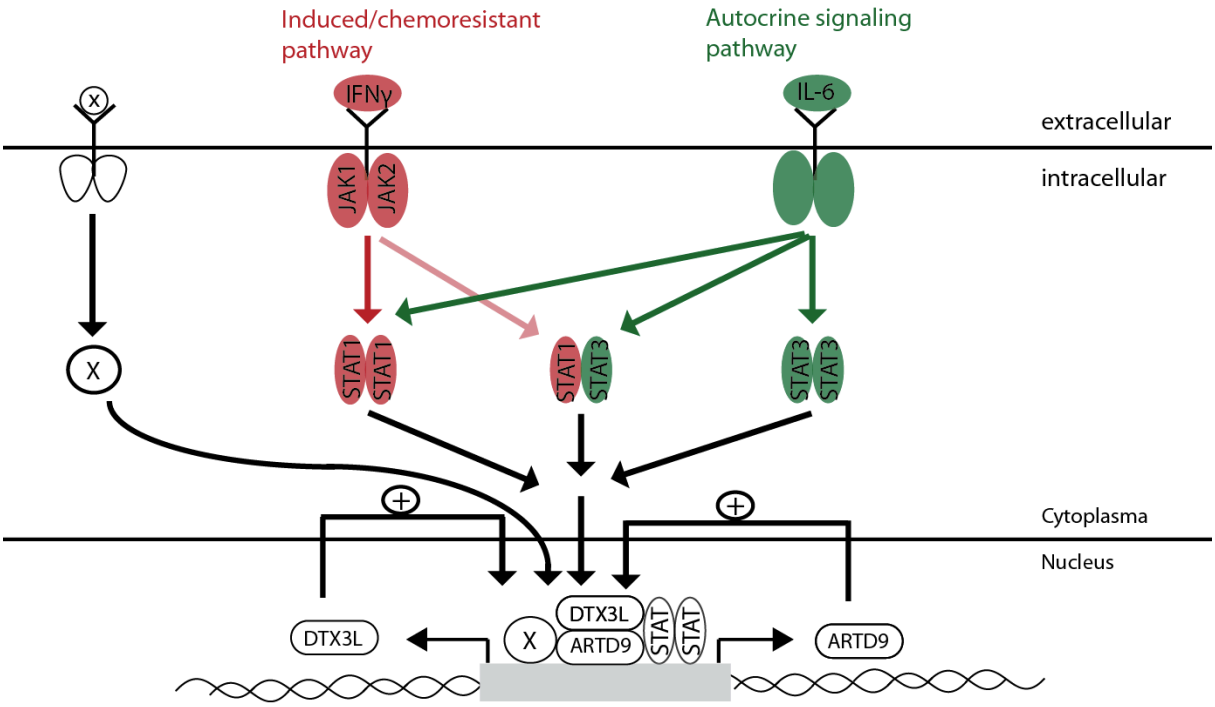

Figure 20: Different ways of regulating DTX3L and ARTD9 expression. The expression of DTX3L and ARTD9 is regulated by a various pathways: an IL6/JAK1-STAT-1:STAT-3-signaling pathway, by IFNγ/JAK1-STAT-1:STAT-1, through the two proteins themself and also unknown factors.

5.2 DTX3L and ARTD9 repress expression of the tumor suppressor IRF1 and mediate cell proliferation, survival and chemo-resistance in PCa cells in a STAT-1 dependent manner

It has been recently shown that ARTD9, a target gene of IFNγ/STAT-1 and IL6/STAT-3 signaling, acts as a crucial modulator of STAT-1-signaling by repressing the anti-proliferative and pro-apoptotic IFNγ-STAT-1-IRF1-p53 axis in high-risk HR-DLBCL [72]. This recent study demonstrated that ARTD9 influences the nuclear activity of the transcriptionally repressive isoform STAT-1β thereby tipping the antagonistic balance between STAT-1 dimers activating transcription (STAT-1α) and STAT-1 dimers repressing transcription (STAT-1β) [72]. ARTD9 can bind to the *IRF1*-promoter and together with STAT-1β inhibits the transcription of the *IRF1* gene, thereby counteracting the IFNγ-dependent anti-proliferative and pro-apoptotic IFNγ-STAT-1-IRF1-p53 axis in high-risk HR-DLBCL [72].
The current study demonstrates that DTX3L and ARTD9 act together as repressors of tumor suppressor IRF1 in mPCa. siRNA knockdown experiments in the PC3 cells revealed that both DTX3L and ARTD9 inhibit the expression of IRF1 in mPCa cells. Subsequent experiments also revealed that IRF1 does indeed inhibit proliferation of mPCa cells. On the other hand, IRF1 does not significantly inhibit survival of mPCa cells (data not shown), strongly indicating that other STAT-1-dependent target genes are involved and/or required for the DTX3L/ARTD9-mediated effects on survival of mPCa cells. Future studies will therefore be required to identify the target genes involved in these processes and elucidate the exact molecular mechanisms.

In line with the previous study [72] the current study shows that the observed DTX3L and ARTD9 mediated cell proliferation, chemo-resistance and survival is also dependent on STAT-1 in mPCa. Conversely, control experiments revealed that STAT-1 and ARTD8 do not act together in the same signaling pathway. Together these results suggest that both DTX3L and ARTD9 mediate cell proliferation, survival and chemo-resistance of mPCa cells in a STAT-1-dependent manner in the presence or absence of IFNγ and/or Docetaxel. These effects are at least in part mediated through the inhibition of the pro-apoptotic and/or anti-proliferative IFNγ-STAT-1-IRF1-axis (very likely together with STAT-1β).

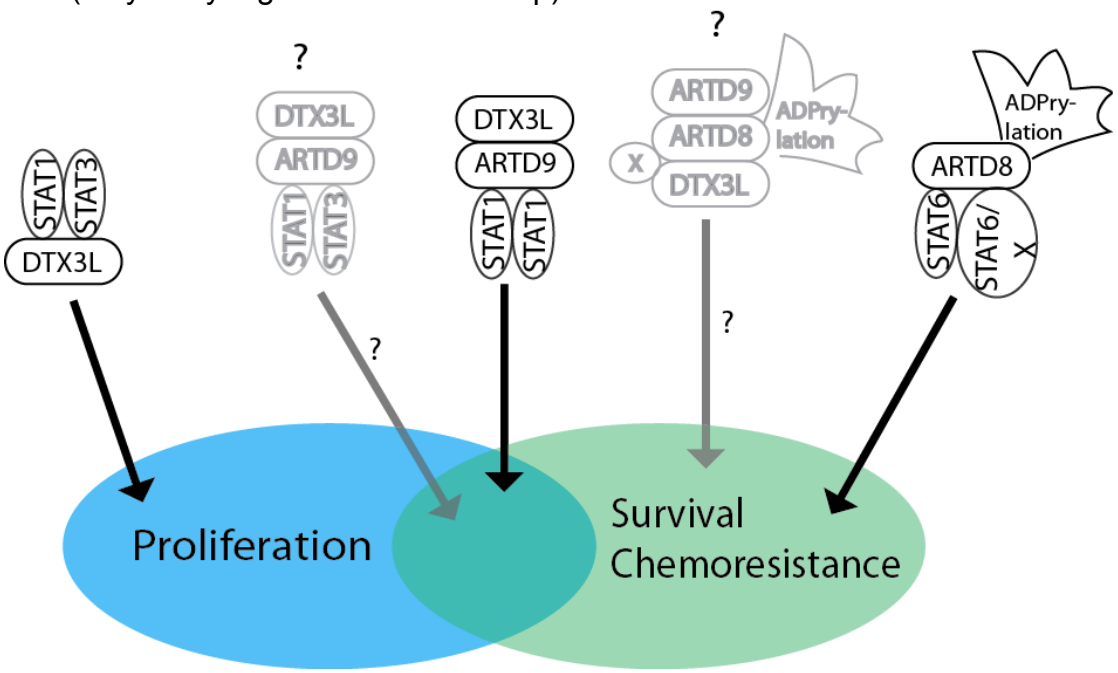

Figure 21: Possible protein complexes influencing proliferation, survival and chemo-resistance in PC3 and DU145 cells.

IRF1 mediates pro-apoptotic effects in cancer cells mainly in a cell type- or context-specific manner [99, 36]. Future studies will be therefore required in order to identify the target genes involved in these processes and elucidate the exact molecular mechanisms.

5.3 Crosstalk between STAT-1/ARTD9/DTX3L and ARTD8 mediated signaling pathways

This study revealed that DTX3L and ARTD9 act together in the same STAT-1-dependent signaling pathways in cell proliferation, survival and chemoresistance. On the other hand, ARTD8 and ARTD9/DTX3L regulate different signaling pathways but synergistically act together in cell proliferation and survival.
A recent study in mice provided first evidence that ARTD8 functions as a STAT-6-specific co-regulator of IL4-mediated gene expression [61]. It shows an involvement of STAT-6 in IL4-induced proliferation and protection of B-cells against apoptosis following irradiation or growth factor withdrawal [61]. Also STAT-6 has been recently shown to act as a survival factor and enhances metastatic prostate cancer progression [100]. Although no clear correlation between STAT-6 expression or

STAT6 activity and ARTD8 could be observed in the analyzed metastatic prostate cancer cell lines, it is possible that ARTD8 may act together with DTX3L as a STAT-6-specific survival factor (Figure 21). Alternatively, ARTD8 might act together with DTX3L independently of STAT-6 signaling in these cell lines. Indeed, a recent study provided evidence that ARTD8 promotes the JNK2-dependent survival of myeloma cells by binding and inhibiting c-Jun N-terminal kinase (JNK)-1 kinase activity, independently of IL4/STAT-6 [46].
Together, the current study strongly suggests a functional crosstalk between ARTD8, DTX3L and ARTD9 in proliferation and survival while DTX3L and ARTD9 very likely regulate the same signaling pathways proliferation and survival and chemo-resistance of mPCa.

5.4 Migration

The shown migration analyses revealed that DTX3L but not ARTD9 or ARTD8 mediates cell migration in the metastatic prostate carcinoma cell lines PC3 and DU145 (Figure 18). The effect on migration observed in siARTD9 knockdown cells was minor when compared with siMock and siDTX3L knockdown cells, and most likely due to the lower DTX3L levels in siARTD9 cells.
Recent studies provided preliminary evidence that STAT-1 is also involved in migration of metastatic prostate cancer cells [87, 88]. Moreover, besides its role in mediating tumor survival and growth, STAT-3 plays a crucial role in tumor migration, invasion and metastasis [46, 26]. The current study suggests that the observed DTX3L mediated migration is indeed dependent on STAT1 and STAT3 signaling (Figure 19). No further significant inhibition was observed in siDTX3L/siSTAT-1 double knockdown cells when compared with siDTX3L or siSTAT-1 single knockdown cells, strongly indicating that DTX3L and STAT-1 act in the same pathway(s).
The shown constitutively expressed pSTAT-1-S727 (Figure 14) indicates its possible influence on migration. The control experiments with siIRF1 cells showed no influence of IRF1 on migration. This supports the possible role of pSTAT-1-S727 in migration.
Together, these experiments indicate that pSTAT-1Y701 influences mainly survival and proliferation, which are also sensitive towards IFNγ. On the other hand STAT-1 mediated migration is not dependent on IFNy and might be directly mediated through the combined action of pSTAT-1-S727 and pSTAT-3. However it remains to be investigated in future studies whether STAT-1 and STAT-3 act indeed as heterodimer in migration.

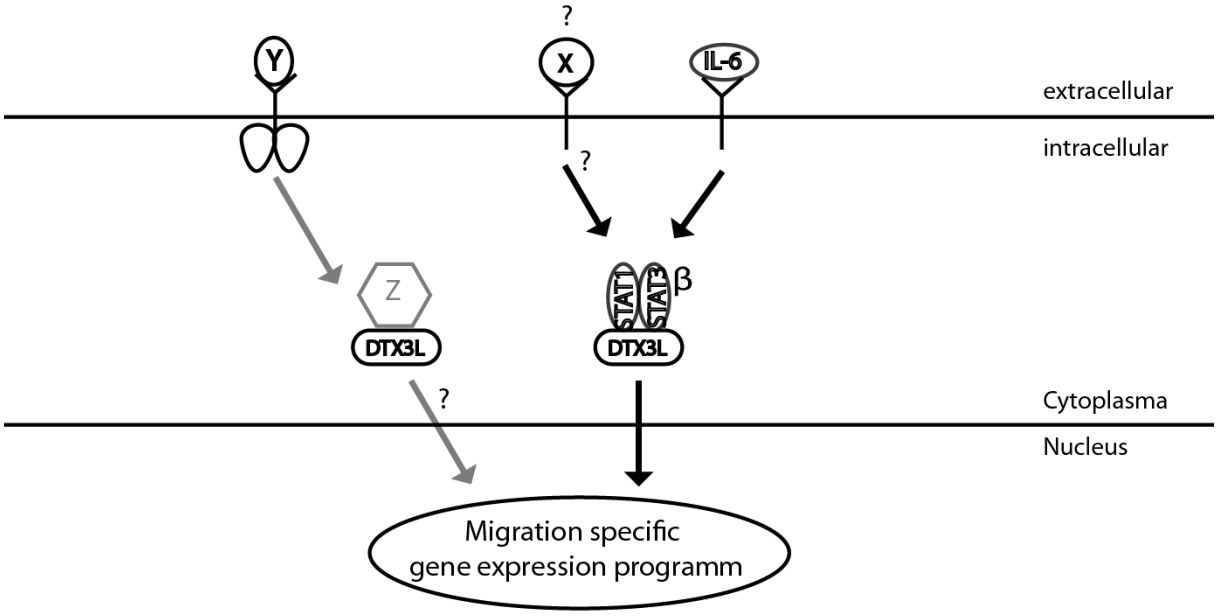

Figure 22: Possible interaction of DTX3L, STAT-1 and STAT-3 in migration of PC3 and DU145 cells.

6 Conclusion and outlook

The E3 ubiquitin ligase DTX3L and the macrodomain-containing mono-ADP-ribosyltransferases ARTD8 and ARTD9 were here identified as novel oncogenic survival factors in androgen-independent mPCa cells. However, contrary to the situation in HR-DLBCL, the DTX3L/ARTD9-mediated effects on survival observed in the mPCa cell lines used in this study are only partially dependent on IRF1 in these cells. Thus, the DTX3L/ARTD8 and DTX3L/ARTD9 target genes, which act together with IRF1 in mediating survival and/or proliferation, remain to be identified in future studies.

In addition to their regulatory roles in STAT-1-mediated chemo-resistance, both DTX3L and ARTD9 could also be directly involved in editing or inhibiting the IFNγ-dependent host immune response against tumor cells through the termination of IFNγ-mediated gene expression and the inhibition of the extrinsic IFNγ-induced anti-proliferative and pro-apoptotic STAT-1-IRF1-p53-axis. Alternatively, the observed crosstalk between DTX3L/ARTD9 and ARTD8 in absence of IFNγ strongly indicates that DTX3L/ARTD9 and ARTD8 act independently of IFNγ-mediated signaling in cell proliferation and survival.

The *in vitro* study shown here indicates that DTX3L together with STAT-1 might also be required for the metastasis and dissemination of metastatic PCa cells *in vivo*. Thus, further studies need to be carried out to determine whether simultaneous ectopic co-overexpression of DTX3L, ARTD8 and/or ARTD9 in xenograft prostate tumors *in vivo* cause Docetaxel-resistance and enhanced metastasis *in vivo*.

Finally, this data provides first evidence for a crossstalk between mono-ubiquitin-ligase(s) and mono-ADP-ribosyltransferases that mediates proliferation and survival in mPCa. It suggests that these processes might be tightly regulated by mono-ADP-ribosylation and mono-ubiquitination. However, the exact molecular mechanisms and the exact functional roles of its mono-ADP-ribosylation activity underlying the observed crosstalk remain to be elucidated in future studies.

Altogether, this study suggests that the combined targeted inhibition of STAT-1, ARTD8, ARTD9 and/or DTX3L could increase the efficacy of chemotherapy or radiation treatment in prostate and other high-risk tumor types with an increased STAT-1-signaling. For instance, the combination of classical therapeutic drugs with highly ARTD8 or DTX3L-specific inhibitors and drugs specifically targeting STAT-1 or the macrodomains of ARTD9 might provide a novel therapeutic strategy to increase the sensitivity of PCa cells towards classical therapy. This could pave the way for the development of novel personalized therapeutic strategies for patients suffering from aggressive PCa.

7 Figures and Tables

7.1 Figures

7.2 Tables

8 References

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